Serotonergic augmentation strategies; possibilities and limitations

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Voor mijn vader
Harm Jongsma

die mij geleerd heeft
om vol vertrouwen
de uitdagingen die het leven
te bieden heeft
aan te gaan

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CHAPTER 1

General introduction

M.E. Jongsma, M.C.G. van der Hart, J.I. Udo de Haes, T.I.F.H. Cremers, B.H.C. Westerink, J.A. den Boer and F.J. Bosker

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1 Introduction

The term depression is used for a variety of affective disorders sharing feelings of depressed mood but which otherwise consist of a highly variable set of symptoms. This heterogeneity in combination with a high co-morbidity of other psychiatric disorders complicates diagnosis and treatment considerably, but is also a confounding factor when investigating the pathophysiology of depression.

Research into the pathophysiology and pharmacotherapy of depression more or less started by serendipity in the late 1950s when an attentive physician noted that the antitubercular drug isoniazide also improved mood in some of the patients. It appeared that isoniazide interfered with the metabolism of monoaminergic neurotransmitters through inhibition of the enzyme monoamine oxidase (MAO). The idea that depression is caused by a monoamine deficiency in the brain is a key element of the monoamine hypothesis (Schildkraut, 1965), and still forms the basis of (rational) antidepressant drug development. Although far from offering a satisfactory explanation of the neurobiological origin of depression the monoamine hypothesis is still used in drug development, simply because psychiatric research did not come up with better alternatives.

The improvement of monoaminergic transmission is also the leading theme in the present thesis. It is generally believed that the therapeutic effect of a majority of currently prescribed antidepressants is related to the enhancement of serotonin levels in the brain. How this increase exactly leads to an antidepressant response is still far from clear, but as detailed in the following section, it can be used as a conceptual framework for further research.

The need for improved efficacy is emphasized by the moderate effectiveness of antidepressants (Kirsch et al., 2002) compared to placebo, the considerable non-response rate and the late onset of action. In terms of pharmacology, the enhanced therapeutic effect might be realized through an additional increase, or augmentation, of the neurotransmitter serotonin. Although it is a rather crude approach for a complex disease like depression, this offers a rational starting point to improve antidepressant treatment.

2 Neurobiology/pathophysiology of depression

2.1 Monoamine hypothesis of depression

The monoamine hypothesis of depression does not only propose the crucial involvement of monoamines in the therapeutic effects of antidepressant drugs but also suggests that depression is directly related to decreased monoaminergic transmission. It is based on the observations that depletion of monoamine stores in the brain by reserpine induced depressive symptoms, while increasing extracellular monoamine levels in the brain appeared to be effective in several forms of depression (Schildkraut, 1965).

The monoamine hypothesis has not been without criticism, but there are new data that could fit in. Many of them come from positron emission tomography (PET) studies. By using selective radioligands evidence was found for reduced pre- and postsynaptic 5-HT_{1A} receptor binding in depression. Drevets et al. (1999) demonstrated that the mean 5-HT_{1A} receptor binding potential (BP) was reduced in unmedicated depressed patients relative to healthy controls using [^{11}C] WAY-100635. These data are consistent with those of Sargent et al. (2000). However, a subgroup of the subjects were scanned both pre- and post-paroxetine treatment, and 5-HT_{1A} receptor BP did not significantly change in any area. A recent PET study with [^{11}C]DASB, a selective radioligand for the 5-HT transporter (5-HTT), in patients suffering from major depressive episodes (MDE) also investigated the contribution of another factor associated with depressed mood, namely the presence of dysfunctional attitudes and the relationship thereof with the 5-HTT binding potential (Hervas et al., 2001). Dysfunctional attitudes are negatively biased assumptions and judgements about the world and oneself and constitute a negative cognitive interpretative bias of the future. Most studies have investigated the relationship with depression as a syndrome and ignored the presence of dysfunctional attitudes. Interestingly, no differences in 5-HTT binding potential (BP) were found among the entire sample of depressed patients compared to healthy controls. However, depressed patients with high regional 5-HTT BP (up to 21%) had higher levels of dysfunctional attitudes. It has been suggested that an increased density of the 5-HT transporter (5-HTT) may lead to increased 5-HT clearance from the synapse, thus leading to a reduced availability of synaptic 5-HT. A PET study using the 5-HTT ligand [^{11}C](+)McN5652 has demonstrated that the distribution volume ratio of this PET ligand was larger in left prefrontal cortex and right cingulate cortex of depressed patients compared to controls (Reivich et al., 2004). These findings suggest that 5-HTT sites may be increased in frontal and cingulate cortical areas of patients suffering from major depression. Such changes might not lead to depression per se, but to a negative cognitive bias, which could pave the way

for the development of personality disorders and other psychiatric syndromes. According to these neuroimaging studies serotonin is likely to play a role in the neurobiology of depression in at least a subgroup of patients.

Another approach that supports the monoamine hypothesis is depletion of the serotonin precursor tryptophan. Studies using this paradigm support the role of serotonin in the modulation of mood, as witnessed by the ability of tryptophan depletion to lower mood (Delgado et al., 1990; Miller et al., 1992). Smith et al. (1999) studied the effect of relapse, following tryptophan depletion, on cognitive function of depressed patients. They reported an attenuation of the task (verbal fluency) related activation in the anterior cingulate during relapse, which was correlated to an increase in depressive symptoms. These results are in agreement with previous studies using a similar paradigm. For instance, inhibition of 5-HT synthesis by p-chlorophenylalanine (Shopsin et al., 1975; Shopsin et al., 1976) or L-tryptophan depletion (Delgado et al., 1990; Miller et al., 1992) caused a relapse of symptoms in depressed patients that were successfully treated with SSRIs (Bell et al., 2001; Reilly et al., 1997). These studies clearly demonstrate that serotonin plays a crucial role in the therapeutic effect of SSRIs.

Studies into polymorphisms of the genes that code for tryptophan hydroxylase (TPH), the rate limiting enzyme in the synthesis of serotonin, have lead to mixed results. A polymorphism of the tryptophan hydroxylase (TPH1) gene, which is expressed both peripherally and centrally, was found to be associated with suicidal behavior and not with depression per se (Bellivier et al., 2004). More encouraging results were reported for two different centrally expressed TPH2 gene polymorphisms, both displaying a significant association with major depression (Zhang et al., 2005; Zill et al., 2004a) and in one case also suicidal behavior (Zill et al., 2004b).

The studies cited above clearly support the monoamine theory of depression, but evidence is also accumulating against a direct relation between depression and a monoamine deficiency (Delgado, 2000; Hirschfeld et al., 2000). For example, current evidence concerning serotonin does not imply depression but rather aggressiveness, failing impulse control and violent suicide as directly related to impaired brain serotonergic function (Russo et al., 2003). It is also worthy to note that tianeptine, a reuptake enhancer, also has antidepressant effects. Moreover, other reuptake inhibitors such as cocaine do not possess antidepressant activity.

Notwithstanding the arguments raised against a direct relation between depression and a monoamine deficiency, it is concluded that there is sufficient evidence to support the use of the monoamine hypothesis as conceptual framework, in particular for pharmacotherapy.

3 Serotonergic system

Since it was discovered that serotonin has a function as neurotransmitter in the central nervous system, the serotonergic system has been the target of scientific research, which has resulted in a vast amount of information regarding its anatomy, physiology and pharmacology.

3.1 Anatomy

Serotonergic neurons are present throughout the brain, but most cell bodies are clustered in nine serotonergic nuclei located in the brain stem. The majority of serotonergic innervations are derived from the two largest nuclei, the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) (Dahlstrom and Fuxe, 1964), which innervate important brain structures such as the cortex, hippocampus and hypothalamus. These brain areas are part of the limbic system, which is involved in emotional behavior and its dysfunction is thought to underlie the pathology of several affective disorders.

Most brain areas are jointly innervated by both nuclei, but exceptions are the prefrontal cortex and the dorsal hippocampus, which are predominantly controlled by the DRN and MRN, respectively (McQuade and Sharp, 1997). In addition to the classical view of nuclei controlling terminal release, projections from terminal areas also appear to exert some control on activity of the raphe nuclei, creating a subtle balance between the brain stem nuclei and the brain areas they project to (Bosker et al., 2001).

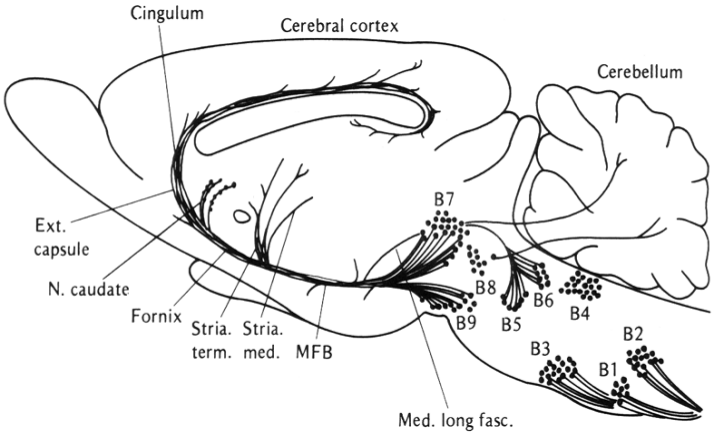

Fig. 1. *Overview of the main serotonin-containing pathways in the rat central nervous system.*

3.2 Physiology

Serotonin is present both centrally and peripherally. Because serotonin is unable to pass the blood brain barrier under normal conditions, it has to be synthesized from the precursor tryptophan, which can only be derived from the diet. Plasma tryptophan is actively transported by a non-selective carrier mechanism and has to compete with other large neutral amino acids such as tyrosine, phenylalanin, (iso)leucine and valine. Consequently, the amount of tryptophan transported into the brain does not only depend on blood tryptophan levels but also on the levels of other circulating amino acids (Dahlstrom and Fuxe, 1964).

After transport over the blood brain barrier, tryptophan is converted into 5-hydroxy tryptophan (5-HTP) by the specific enzyme tryptophan hydroxylase, which is located both in the cell body and projection area. Finally, serotonin is synthesized from 5-HTP by the non-specific enzyme aromatic amino acid decarboxylase (AADC), which is localized in the axon terminals of all monoaminergic neurons. Normally, tryptophan hydroxylase is unsaturated and hence, the amount of serotonin synthesized solely depends on the levels of tryptophan in the brain. But if tryptophan levels rise, the enzyme gets saturated and will become the rate-limiting factor (Carlsson and Lindqvist, 1978; Westerink and Devries, 1991).

When released from the neuron, the action of serotonin can be terminated by metabolism or reuptake into the presynaptic neuron. Serotonin is metabolized by monoamine oxidase into 5-hydroxyindolacetaldehyde, which subsequently can be oxidized by aldehyde dehydrogenase into 5-hydroxy indolacetic acid (5-HIAA), or reduced into 5-hydroxytryptophol. Oxidation is the main metabolic route, which makes 5-HIAA the predominant metabolite of serotonin. 5-HIAA itself is actively transported out of the brain, so central levels of 5-HIAA can be used as a marker of serotonergic metabolism. Once released, serotonin can be transported back into the presynaptic neuron by a specific carrier protein, which also is the main target of selective serotonin reuptake inhibitors (SSRIs).

3.3 Pharmacology

Until now, 14 different receptor subtypes have been discovered, which are classified in 7 different families. By increasing extracellular serotonin, antidepressants act as indirect agonists on all 14 receptors, of which some are known to control serotonergic activity through an inhibitory feedback mechanism. Antagonists of such autoreceptors are able to augment the serotonergic response of antidepressants, demonstrating the role of these receptors in the pharmacology of antidepressants.

3.3.1 5-HT$_{1A}$ receptors

When activated, the G-protein linked 5-HT$_{1A}$ receptor stimulates the opening of potassium channels, thereby decreasing the ability of serotonergic neurons to depolarize. This causes a reduction of neuronal activity and as a result, serotonergic release is also diminished.

5-HT$_{1A}$ autoreceptors are localized on the cell body, effectively controlling firing rate, serotonin release and synthesis (Hutson et al., 1989; Sharp et al., 1989; Sprouse and Aghajanian, 1987). Electrophysiology studies have demonstrated a strong reduction of the firing rate after administration by 5-HT$_{1A}$ receptor agonists, including the endogenous ligand serotonin (Sprouse and Aghajanian, 1987). Similarly, microdialysis studies have shown a diminished release of serotonin following the administration of 5-HT$_{1A}$ receptor agonists (Hutson et al., 1989). The effects were completely blocked by 5-HT$_{1A}$ receptor antagonists, indicating the involvement of 5-HT$_{1A}$ receptors.

3.3.2 5-HT$_{1B/1D}$ receptors

Species differences in pharmacological profile of 5-HT$_{1B}$ receptors have been the reason for confusion with respect to their nomenclature. Receptors could be assigned both 1B and 1D, depending on the property observed. Nowadays, a unifying nomenclature exists which is used by most scientists. In order to avoid any ambiguity, both receptor subtypes will be described comprehensively.

The 5-HT$_{1B}$ receptors of human, guinea pig and most other species are very similar with respect to their structure and pharmacological profile. In contrast, rat and mice 5-HT$_{1B}$ receptors, in spite of having a comparable primary structure, display a substantially different pharmacological profile. With respect to their function in the brain, these receptors are all thought to be species homologues. 5-HT$_{1B}$ receptors are localized on the axon terminals, directly controlling serotonin synthesis and release. Administration of 5-HT$_{1B}$ receptor agonists decreases serotonin release in terminal regions, which can be blocked by 5-HT$_{1B}$ antagonists (Bosker et al., 1995; Hjorth and Tao, 1991).

Using the new nomenclature, 5-HT$_{1D}$ receptors are species homologues with respect to their structure, functionality as well as their pharmacological profile. However, the amount of 5-HT$_{1D}$ receptors in the brain does differ between species; the rat for example has a relatively low density of these receptors. The 5-HT$_{1D}$ receptor is also a G protein coupled receptor, which upon activation exerts its inhibitory action by a decrease of the second messenger cAMP. In contrast with 5-HT$_{1B}$ receptors, 5-HT$_{1D}$ receptors are localized in both terminal regions and on the cell bodies in the raphe nuclei. Although this receptor is scarcely involved in control of serotonergic

release in terminal regions, it does seem to have an autoreceptor function in the raphe regions (Sprouse et al., 1997; Starkey and Skingle, 1994).

3.3.3 5-HT_2 receptors

The 5-HT_2 receptor family consists of three subtypes, using the same second messenger system, the inositol triphosphate (IP_3) pathway, to activate protein kinase C. Moreover, all 5-HT_2 receptor subtypes have an inhibitory role. Whereas the 5-HT_{2B} receptor is merely found in the periphery, 5-HT_{2A} and 5-HT_{2C} receptors are predominantly located in the brain (Barnes and Sharp, 1999; Hoyer et al., 1994). Although both 5-HT_{2A} and $_{2C}$ receptors are thought to be localized in terminal regions, their agonists not only decreased terminal serotonin release, but also diminished the firing rate of the serotonergic neurons. The effects on release and firing rate could, however, not be blocked with receptor antagonists, indicating that 5-HT_2 receptors are not directly involved in the release of serotonin (Moret and Briley, 1997).

4 Neuropharmacology of antidepressants

4.1 A short history

The antidepressant effect of the antitubercular drug isoniazide was attributed to its ability to increase monoamine levels in the brain through inhibition of the enzyme monoamine oxidase. Monoamine oxidase inhibitors (MAOis) appeared to be effective antidepressants, but they had serious side effects not in the least because they also inhibited monoamine oxidase-A in the liver. Incidentally this has led to hypertensive crisis (tyramine effect), which could be fatal especially for patients of advanced age.

The next generation of antidepressants, the tri- and tetracyclics (TCAs) also increased monoamine levels in the brain albeit via a different mechanism. Originally developed to treat schizophrenia, TCAs appeared to block the reuptake of monoamines, which also resulted in increased monoamine levels in the brain. Unfortunately, this generation of antidepressants also had severe side effects, which was attributed to their lack of selectivity. This has led to the development of selective serotonin reuptake inhibitors (SSRIs). Their side effect profile appeared relatively benign in comparison with TCAs and MAOis. The image of efficacious and save drugs has strongly contributed to their current status in antidepressant pharmacotherapy.

4.2 Desensitization hypothesis

A large body of evidence indicates that SSRIs increase extracellular levels of serotonin by preventing this neurotransmitter to be taken up again after being released (Fuller, 1994). Although this pharmacological effect occurs immediately, the therapeutic response is typically delayed for several weeks, suggesting that adaptive changes at the cellular level are required to attain the full antidepressant effect. This observation is the basis of the desensitization hypothesis raised by Blier et al. (1987a). Using single cell recordings in rats it was found that acute administration of antidepressants decreased serotonergic cell firing through the activation of 5-HT autoreceptors, but tolerance developed upon chronic antidepressant treatment, which was attributed to desensitization of 5-HT autoreceptors (Chaput et al., 1986). Because of the striking similarity between onset of antidepressant action and the time required for desensitization of the autoreceptors, it was proposed that these effects were connected. In addition to the electrophysiology experiments, microdialysis studies have shown that chronic treatment also reduced the inhibitory effect of 5-HT agonists on serotonin release, further supporting the desensitization hypothesis (Chaput et al., 1986; Invernizzi et al., 1994; Kreiss and Lucki, 1995).

As a consequence of 5-HT reuptake inhibition, SSRIs increase extracellular 5-HT levels and one would expect this effect to become more prominent following chronic treatment, due to the restoration of 5-HT firing rate. This is supported by microdialysis studies, combining SSRIs with specific antagonists of 5-HT autoreceptors in order to mimic desensitization of 5-HT autoreceptors, which indeed resulted in further increases of extracellular 5-HT levels (Hjorth, 1996; Invernizzi et al., 1997).

Although Blier's desensitization hypothesis is now broadly accepted, some marginal notes have to be made. Desensitization has been postulated as a general process, but in fact this phenomenon has only been truly established for the presynaptic 5-HT_{1A} autoreceptors in the raphe nuclei. Evidence for the desensitization of postsynaptic 5-HT_{1A} receptors and other 5-HT receptor subtypes appeared to be far less convincing, reducing the general validity of the desensitization hypothesis. In addition, not all antidepressants diminish the function of 5-HT_{1A} autoreceptors following chronic treatment, suggesting that desensitization is not essential in order to achieve the antidepressant effect.

4.3 Antidepressant drug targets

SSRIs inhibit the reuptake of serotonin throughout the brain, but the effect varies considerably between brain areas (Fuller, 1994). Consequently, investigating the regional effects of antidepressants might give an impression which brain areas are involved in depression and in the

antidepressant effect of SSRIs. Neuroimaging techniques have allowed us to investigate the human brain in a non-invasive manner, thereby greatly expanding our knowledge of the neuronal networks involved in both pathophysiology and pharmacotherapy of psychiatric disorders.

Affective behavior and control of emotions is generally related to the limbic system, which consists of various brain areas including the amygdala, prefrontal cortex, hippocampus and hypothalamus. Problems with control of emotional behavior associated with affective disorders such as depression have been attributed to dysfunctions of the limbic system.

Evaluation of the neuropharmacological effects of psychoactive drugs did, however, not lead to unequivocal results, with varying outcomes depending on the imaging technique used.

Microdialysis is an excellent technique to study regional effects of antidepressants on extracellular serotonin levels, but information is restricted to the brain region where the dialysis probe is located. The changes of extracellular serotonin levels might have different postsynaptic consequences depending on the brain region being investigated. By studying such effects in several brain areas simultaneously, cellular markers of neuronal activity, for instance the immediate early gene c-fos, might provide a useful extension of the (presynaptic) microdialysis data.

4.3.1. Neuroimaging

4.3.1.1 Depression

Most brain imaging studies conducted in patients with major depression episodes (MDE) have been able to identify abnormalities associated with MDE, e.g. (Dolan et al., 1992; Drevets, 2000). In the vast majority of studies conducted in the eighties and early nineties, correlates were sought of the syndrome of major depression, without paying much attention to specific brain regions related to symptom components of MDE. There is, however, a large inter-individual variability in severity and psychopathological features associated with MDE. A more fruitful approach would be to search correlates between processes in the brain and subcomponents of MDE, such as motivation, depressed mood, dysfunctional attitudes and sleep disturbances, instead of trying to find neuronal correlates for depression as a syndrome.

Several studies found that severity of depression is positively correlated with regional cerebral glucose uptake, as measured with [^{18}F]-fluorodeoxyglucose positron emission tomography ([18]-FDG PET). These studies reported positive correlations between depression-severity and brain regions such as the bilateral medial frontal cortex, anterior cingulate, right dorsolateral hippocampus, cingulate and other paralimbic areas (Osuch et al., 2000; Videbech et al., 2002).

There is more controversy with respect to studies that report negative correlations between depression severity and regional brain activity (for references, see Milak et al. (2005). A possible explanation could be that the diversity of clinical manifestations of MDE may obscure associations with specific brain regions. In a recent study, Milak and collegues (2005) have investigated the association between different psychopathological clusters of the Hamilton Depression Rating Scale (HDRS) and resting glucose metabolism using [^{18}F]-FDG PET. They found distinct correlations between three HDRS factors and regional glucose metabolism. The first factor, psychic depression showed a positive correlation with metabolism in the basal ganglia, thalamus and cingulate cortex. The second factor, sleep disturbance, showed a positive correlation with metabolism in limbic structures and basal ganglia, and the third factor (loss of motivation) was negatively correlated with parietal and superior frontal cortical areas. Interestingly, this study shows that positive correlations with aspects of depression severity are subcortical ventral, ventral prefrontal and limbic structures, whereas negative correlations are found in dorsal cortical areas (Milak et al., 2005).

4.3.1.2 Antidepressants

Studies in healthy volunteers using acute serotonergic challenges such as fenfluramine or SSRIs have shown changes in a discrete network of brain regions. Most studies have found relative decreases in activation in the thalamus and (medial) temporal areas, including hippocampus and amygdala, and increases in frontal areas, including the anterior cingulate (Cook, Jr. et al., 1994; Geday et al., 2005; Kapur et al., 1994; Mann et al., 1996; Meyer et al., 1996; Smith et al., 2002b). These areas show abnormal activation patterns in depressed patients, and in most studies a reversal of pretreatment abnormalities in brain activity is seen after SSRI treatment. These studies also found evidence for a role of the anterior cingulate in the pathology and treatment of depression (Brody et al., 1999; Kegeles et al., 2003; Mayberg et al., 2000; Oquendo et al., 2003; Smith et al., 2002a). Studies in patients with impulsive- aggressive personality disorder (Siever et al., 1999) and panic disorder (Meyer et al., 2000) reported abnormal fenfluramine-induced changes in frontal regions and the parietal-superior temporal cortex, respectively. In patients with obsessive-compulsive disorder (OCD) increased baseline activity is found in the cingulate cortex, orbitofrontal cortex, caudate nuclei and thalamus when compared to healthy controls, and SSRI-related clinical improvement appeared to correlate with a decrease in these regions (Perani et al., 1995; Saxena et al., 1998; Saxena et al., 1999; Saxena et al., 2002).

4.3.2 Expression of immediate early genes

Expression of immediate early genes, assessed either via mRNA or corresponding protein, has been used as index for the postsynaptic effects of antidepressants. Although far from being a selective measure for antidepressant effects it may give some idea which neuronal networks in the brain are initially activated by antidepressants. Assessment of the chronic effects is probably more difficult because tolerance to this type of gene expression easily develops (Veening et al., 1998). A study by Miyata et al. (2005) showed that fluoxetine and reboxetine similarly increased Fos-immunoreactivity (Fos-ir) in the shell of the nucleus accumbens (Nacc), however activation patterns were quite different in cortical areas and central nucleus of the amygdala (CeA). The authors note that activation of the "limbic" nucleus accumbens by both fluoxetine and reboxetine is in accordance with the compounds' comparable efficacies in treating symptoms of anhedonia. Conversely, their differential efficacy regarding other symptoms of depression may relate to the different activation pattern seen in the other areas. A tendency to increase Fos-ir in NAcc and CeA was also seen for citalopram, which became significant when the SSRI was co-administered with the 5-HT_{1A} receptor antagonist WAY 100.635 (Jongsma et al., 2002). Augmentation was also seen in paraventricular nucleus of the hypothalamus (PVN), ventromedial hypothalamus and dorsolateral striatum. It has been hypothesized that the mood-stabilizing effect of antidepressants is achieved by their action on the limbic-hypothalamic-pituitary-adrenocortical system (Barden et al., 1995). Given the strong augmentation seen in Nacc, CeA and PVN, co-administration of a 5-HT_{1A} receptor antagonist with an SSRI seems an attractive proposition. On the other hand co-administration of WAY 100.635 may exacerbate SSRI induced sexual dysfunction (de Jong et al., 2005). Brain areas in which no Fos-ir was found in both control and any of the treated animals included the median raphe nucleus and the hippocampus. The latter is in accordance with a study by Tordera et al. (2003), which has assessed the expression of two early genes following combinations of paroxetine with different selective 5-HT_{1A} receptor antagonists. Augmentation was seen in several cortical areas and caudate putamen. However, differences in activation pattern were also noted between the antagonists, which had not been apparent when measuring extracellular 5-HT levels.

In conclusion, although many questions are left unanswered and some discrepancies have been noted, literature data from both neuro-imaging and early gene expression converge to the involvement of both cortical (anterior cingulate) and limbic areas (CeA, Nacc, dorsal hippocampus).

4.4. Pre- vs. postsynaptic effects

It has always been assumed that serotonergic release in the projection areas is mainly controlled by autoreceptors located presynaptically. Consequently, the therapeutic effect of antidepressants is generally attributed to desensitization of these receptors. However, evidence is accumulating that postsynaptic 5-HT_{1A} receptors are also involved in the control of firing rate and release of serotonergic neurons, and that their function might also change following chronic antidepressant treatment. Besides, SSRIs may be considered as indirect agonists at all fourteen 5-HT receptor subtypes. Most of these are located postsynaptically and display different distribution patterns within the brain. Therefore, it is important to have some idea as to the brain areas and (postsynaptic) 5-HT receptors involved in the neurobiology of depression and the effects of SSRIs.

4.4.1 Postsynaptic 5-HT receptors and (side) effects of SSRIs

Most microdialysis studies have assessed the effect of SSRIs on extracellular 5-HT levels, which confines retrieval of information to the presynaptic element of the 5-HT neuron. It is conceivable, however, that 5-HT receptor subtypes differentially distribute the 5-HT signals over the various brain areas. Several attempts have been made to identify the postsynaptic 5-HT receptors that mediate the therapeutic effect and side effects of antidepressants. For instance, the effect of the SSRI fluvoxamine on forced-swimming –induced immobility in mice, a model for depression, has been reported not to involve the 5-HT_{1A} and 5-HT_2 receptor subtypes (Egawa et al., 1995). Similarly de Vry et al. (De Vry et al., 1997) hypothesized that 5-$HT_{1B/1D}$, 5-HT_{2C}, 5-HT_3 and 5-HT_4 receptors may not critically be involved in the therapeutic effects of SSRIs. Conversely, blockade of 5-HT_2 and 5-HT_3 receptors (Berk et al., 2000; Boyarsky et al., 1999), but not 5-$HT_{1B/D}$ receptors (Jongsma et al., 2005) has been shown to reduce sexual dysfunction, suggesting that this side effect of SSRIs is mediated by 5-HT_2 and 5-HT_3 receptors. Moreover, 5-HT_{2C} antagonists, but not 5-HT_{2A}, 5-HT_{1A} or 5-HT_3 antagonists, reduce the anxiogenic-like effect of acutely administered citalopram, as demonstrated by the social interaction test in rats (Dekeyne et al., 2000). Interestingly, the discriminative stimulus (DS) properties of citalopram are mediated by 5-HT_{2C} receptors. The antidepressants mianserin and mirtazapine, both being 5-HT_{2C} antagonists, dose-dependently blocked DS properties of citalopram, suggesting that DS effects are not related to the antidepressant effect of SSRIs per se (Dekeyne et al., 2001). Recently it was reported that antidepressants are functional antagonists at 5-HT_3 receptors, suggesting a hitherto unknown pharmacological mechanism (Eisensamer et al., 2003).

4.4.2 Postsynaptic 5-HT$_{1A}$ receptor mediated long loop type of feedback

Firstly proposed by Blier and coworkers (Blier et al., 1987b; Blier and de Montigny, 1987) postsynaptic 5-HT$_{1A}$ receptors may also play a role in the regulation of 5-HT release. Since then several studies on this item have been published (Bosker et al., 1997; Bosker et al., 2001; Casanovas et al., 1999; Ceci et al., 1994; Celada et al., 2001; Romero et al., 1994). Interestingly, chronic treatment with citalopram via osmotic minipumps reduced postsynaptic 5-HT$_{1A}$ receptor-mediated feedback in the amygdala (Bosker et al., 2001). The notion that both pre-and postsynaptic 5-HT$_{1A}$ receptors may desensitize following chronic SSRI treatment makes augmentation strategies based on blockade of this receptor subtype even more interesting. Infusion of the 5-HT$_{1A}$ receptor antagonist WAY 100.635 into the amygdala via retrograde microdialysis also markedly increased the effect of citalopram on local extracellular 5-HT levels, indicating that 5-HT$_{1A}$ receptor-mediated long loop type of feedback is particular strong in this area.

Chronic treatment with citalopram via osmotic minipumps completely abolished augmentation with WAY 100.635. Clearly this type of feedback must also be taken into account when assessing the chronic effects of SSRIs. In this respect the prefrontal cortex is particularly interesting. Previous studies had already shown that postsynaptic 5-HT$_{1A}$ receptor-mediated long loop type of feedback also exists within the prefrontal cortex (Celada et al., 2001). However, chronic treatment with citalopram did not lead to desensitisation of such postsynaptic 5-HT$_{1A}$ receptors, in fact quite the opposite occurred. Since 5-HTT and 5-HT$_{1A}$ receptor binding had not changed (see chapter 4), other mechanisms such as changes in receptor-G protein interaction (Castro et al., 2003) or effects on processes more downstream should be considered (Hensler, 2002). An increase of sensitivity was also observed when a 5-HT$_{1A}$ receptor antagonist was co-administered systemically (Gundlah et al., 1997; Hjorth and Auerbach, 1999). It is also noteworthy that chronic antidepressant treatment markedly increased the effect of 5-HT$_{1A}$ receptor antagonism on the firing activity of hippocampal CA3 pyramidal neurons, which also suggests an increased sensitivity of local postsynaptic 5-HT$_{1A}$ receptors (Haddjeri et al., 1998). Interestingly, opposite effects on pre and postsynaptic 5-HT$_{1A}$ receptors following chronic antidepressant treatment have been reported by a recent study wherein an increased and decreased agonist stimulated GTPγS binding was found in hippocampus and raphe nucleus, respectively (Castro et al., 2003). Such opposite effects on pre and postsynaptic 5-HT$_{1A}$ receptor-mediated feedback would imply a shift in control of terminal 5-HT release from the autoreceptors to their postsynaptic counterparts, which could be a factor in the clinical efficacy of antidepressants.

5 Augmentation strategies

5.1 Rationale of augmentation strategies

According to the World Health Organization major depression (MD) will become the number one disabling disease in the next decade. This prognosis is even more alarming considering the outcome of a meta-analysis of clinical studies involving the six most widely prescribed antidepressants approved between 1987 and 1999 by the FDA, which suggested that antidepressant treatment is only marginally more effective than placebo (Kirsch et al., 2002). Other worrying aspects of antidepressant treatments are the considerable nonresponse rates (30-40%) and the late onset of action (2-5 weeks). There is thus a need for improved antidepressant treatment and one approach is augmentation strategies. Price (1998) defines augmentation as an increase of therapeutic efficacy of an antidepressant by an additional drug. The latter drug may be devoid of antidepressant properties (Joffe et al., 1996; Price, 1998). Depending on one's position the concept "augmentation strategy" may be interpreted quite differently. Health professionals have combined antidepressants with other drugs in order to improve the treatment of their patients, and may be more inclined to see it as a way to reduce depressive and comorbid symptoms. Neurochemists, neurobiologists and medicinal chemists are more concerned with drugs effects at the molecular level, and how these effects will influence the function of the brain circuits putatively involved in the pathology of depression. They may be more inclined to view "augmentation strategy" as a means to optimize these drug effects. Without direct access to patients they have to rely on animal models and a conceptual framework to predict the therapeutic consequences, both in terms of efficacy and side effects.

In the present thesis, augmentation of the antidepressant response will be approached according to the latter view.

5.2 Limitations and risks of augmentation

Several homeostatic processes are known to counteract the effect of SSRIs on extracellular 5-HT levels; some make use of 5-HT_{1A} and 5-HT_{1B} autoreceptors, while others act via postsynaptic 5-HT_{1A} and 5-HT_{2C} receptors. Another factor that might limit the effect of SSRIs on extracellular 5-HT levels is the availability of the serotonin precursor and essential amino acid tryptophan. Interfering with such homeostatic processes offers the opportunity to further increase the effect of SSRIs on 5-HT levels.

However, the concept of further increasing 5-HT levels has its weaknesses. For instance, fenfluramine is extremely effective in boosting extracellular 5-HT levels and yet it is not the

ideal antidepressant. The compound is both a 5-HT releaser and reuptake inhibitor, which is likely to have differential intra- and extrasynaptic consequences, but this example clearly indicates that solely aiming at increased 5-HT levels may be to crude a measure to rely upon.

Attention should also be paid to unwanted side effects associated with too large increases of central serotonin levels such as the serotonergic syndrome. The syndrome is characterized by restlessness, agitation and confusion and can be fatal on occasion. Although this side effect has been reported in only few case studies, it is clear that there is a limit in increasing extracellular serotonin levels. One may ask to which extent serotonin levels should be increased to improve the antidepressant effect without inducing these side effects.

By using competition studies with PET tracers, changes of dopamine levels in the human brain can readily be assessed. Unfortunately, this does not apply for serotonin (De Haes et al., 2002) making it as yet impossible to connect antidepressant effect and serotonin levels in humans.

5.3 Augmentation with 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonists

It has been argued that the loss of 5-HT autoreceptor function in consequence of chronic antidepressant treatment could be mimicked instantaneously by blocking these receptors with an antagonist. Such diminished function of 5-HT autoreceptors could indeed be demonstrated by microdialysis studies, wherein the increase of extracellular 5-HT elicited by a single dose of an SSRI was augmented by co-administration of a 5-HT$_{1A}$ receptor antagonist (Cremers et al., 2000; Gundlah et al., 1997; Hjorth, 1993; Hjorth et al., 1996; Invernizzi et al., 1992). In addition to the somatodendritic 5-HT$_{1A}$ autoreceptor-mediated feedback, 5-HT release is also controlled by terminal 5-HT$_{1B}$ receptors. Accordingly, simultaneous administration of the putative 5-HT$_{1B}$ receptor antagonist GR 127935 and an SSRI has been shown to augment the effect of the latter on extracellular 5-HT levels (Gobert et al., 1997; Rollema et al., 1996; Sharp et al., 1997).

5.3.1 Clinical studies

Based on solid preclinical research by his group and others, Artigas (Artigas, 1993) has proposed to improve antidepressant efficacy and onset of action by co-administering SSRIs with a 5-HT$_{1A}$ receptor antagonist. Since selective 5-HT$_{1A}$ receptor antagonists were not available for use in humans the combined β-adrenergic/5-HT$_{1A}$ receptor antagonist pindolol was chosen, but for safety reasons the dose had to be based on the compound's much higher potency for β-adrenoceptors. A preliminary study with previously untreated depressed patients suggested indeed an improvement in both latency and efficacy by combined treatment with paroxetine and pindolol (Artigas et al., 1994). Since then many open label and controlled studies with pindolol have

followed, albeit with variable success (for meta-analysis see (McAskill et al., 1998)). Soon it became evident that the observed clinical effects of pindolol co-administration could not readily be explained by complete antagonism of somatodendritic 5-HT_{1A} receptors. Several PET scan studies have been published on pindolol binding in the human brain (Andree et al., 1999; Martinez et al., 2000a; Martinez et al., 2000b; Rabiner et al., 2000a; Rabiner et al., 2000b). The studies agree that pindolol binds to somatodendritic 5-HT_{1A} autoreceptors at the doses used in clinical studies, however receptor occupancy is moderate and highly variable. A microdialysis study in guinea pigs indicated that the dose of pindolol in clinical studies had been far too low to reasonably expect augmentation of extracellular 5-HT levels in humans (Cremers et al., 2001). Moreover preclinical data also indicated that pindolol has agonistic properties at 5-HT_{1A} receptors in the raphe nuclei in vivo (Fornal et al., 1999a; Fornal et al., 1999b; Sprouse et al., 1998; Sprouse et al., 2000). A recent meta-analysis indicated, however, that pindolol was able to significantly accelerate the therapeutic effect of an antidepressant in the first weeks of treatment (Ballesteros and Callado, 2004). Although it is tempting to use the latter as support for Artigas' concept, the evidence from animal and PET studies cannot be denied. Alternative explanations such as a rapid partial desensitization of the 5-HT_{1A} autoreceptors by the "agonist" pindolol and/or antagonism of β-adrenergic receptors seem therefore more likely.

5.4 Augmentation with 5-HT_{2C} receptor antagonists

Recently evidence was presented for a novel augmentation strategy based on 5-HT_{2C} receptor antagonism (Cremers et al., 2004). Augmentation of extracellular 5-HT was observed in rat hippocampus and cortex with citalopram, sertraline and fluoxetine. The effect was at least of a similar magnitude as that seen with 5-HT_{1A} and 5-HT_{1B} receptor antagonists (Cremers et al., 2000). Genetic elimination of these receptors in mice (5-HT_{2C}-knock out mice) also augmented the effects of SSRIs on extracellular serotonin levels in the brain. Disabling the 5-HT_{2C} receptors also resulted in a significantly increased antidepressant-like effect of SSRIs in the tail suspension test. In the schedule induced polydipsia test, an animal model for obsessive-compulsive disorder with predictive value for the onset of action of antidepressants, the selective 5-HT_{2C} antagonist RS 102221 dramatically decreased latency time of paroxetine (Cremers et al., 2002), indicating potential to hasten antidepressant response. Microdialysis experiments using the selective 5-HT_{2C} receptor antagonist SB 242084 did not show tolerance following chronic paroxetine treatment (Cremers et al., 2002). However, several behavioral studies suggest that 5-HT_{2C} receptors desensitize following chronic antidepressant treatment ((Bristow et al., 2000) and references therein). The reason for this discrepancy is unknown. Apparently, adaptation of 5-HT_{2C} receptors critically depends on their location and/or function. Recent microdialysis experiments indicated

that the mechanism underlying the augmentation of SSRIs by 5-HT_{2C} receptor antagonists is rather complex with $GABA_B$ receptors involved (Jongsma et al., 2004) and possibly also α_1 adrenoceptors.

5.4.1 Clinical studies

Several clinical studies have investigated combinations of SSRIs with atypical antidepressants such as mianserin (Ferreri et al., 2001; Maes et al., 1999) or with antipsychotics such as olanzapine (Shelton et al., 2001). For the first combination the goal was a faster onset of action and/or treatment of refractory depression, but the latter combination was aimed at depression with comorbid psychotic features.

Mirtazapine (Remeron®) is a very successful antidepressant, and it is under investigation for its ability to augment the clinical efficacy of SSRIs (Besson et al., 2000). The latter could easily be explained in terms of synergy between the antidepressant effects of mirtazapine and the SSRI. However, mirtazapine and mianserin are potent 5-HT_{2C} receptor antagonists, and when co-administered with citalopram both markedly augmentated the effect of the SSRI on extracellular 5-HT levels (Cremers et al., 2002). It can be speculated that the synergy between these atypical antidepressants and SSRIs connects to this augmentation, however the final word is to the clinical studies with selective 5-HT_{2C} receptor antagonists yet to come.

Olanzapine has also been shown to augment the effects of fluoxetine in the clinic (Shelton et al., 2001). Microdialysis studies have shown that combined administration of fluoxetine and olanzepine enhances extracellular brain levels of dopamine and noradrenaline more than fluoxetine does alone (Zhang et al., 2000). This may be attributed to the prominent 5-HT_{2C} receptor antagonistic properties of olanzapine, since blockade of this 5-HT receptor subtype has been reported to increase extracellular dopamine and norepinephrine in the brain (Millan et al., 2003; Zhang et al., 2000). Recently it was shown that 5-HT_{2C} receptor antagonists also augment the effects of SSRIs on extracellular serotonin (Cremers et al., 2004). Notably, olanzapine did not augment fluoxetine-induced increases of extracellular serotonin, which may be due to the concurrent blockade of α_1-adrenoceptors in the raphe nuclei by olanzapine. This is supported by the notion that augmentation by the specific 5-HT_{2C} receptor antagonist SB 242084 was completely abolished by the α_1-adrenoceptor antagonist prazosine.

5.5 Augmentation with tryptophan

The rate of synthesis of cerebral serotonin depends on the availability of its precursor tryptophan, which might limit the therapeutic efficacy of antidepressants if insufficiently present. Levels of

circulating tryptophan are to a large extent determined by dietary intake and catabolism. Persistently low tryptophan levels may form a risk to develop psychopathologies, including depression, aggressive behavior and impaired impulse control. Following acute depletion of tryptophan similar symptoms may emerge. Depressed patients treated successfully with SSRIs have been reported to suffer from a short-lasting relapse, concomitant with an acute and transient depletion of tryptophan (Delgado et al., 1990) (for review see (Bell et al., 2001; Reilly et al., 1997)), emphasizing that the antidepressant response is dependent on the continuous availability of the 5-HT precursor.

Rodent studies have shown that, in addition to autoreceptor control, 5-HT release strongly depends on precursor availability (Schaechter and Wurtman, 1989; Westerink and Devries, 1991). Trytophan depletion by either a tryptophan free diet or administration of a trytophan free amino acid drink resulted in decreased central 5-HT levels in rodents (Fadda et al., 2000; Lieben et al., 2004). Conversely, increased levels of circulating tryptophan resulted in a higher basal 5-HT release and an increased SSRI induced 5-HT response (Gartside et al., 1992; Perry and Fuller, 1993), emphasizing the need for exploring the use of tryptophan in antidepressant therapy. Because the release of serotonin depends on both autoreceptor control and synthesis, tryptophan may also have merit in augmentation strategies.

5.5.1 Clinical studies

There is increasing evidence that patients treated with antidepressants may suffer from tryptophan depletion. Moreover several studies indicated that the therapeutic effect of an SSRI is critically linked to the availability of tryptophan (Bremner et al., 1997; Leyton et al., 2000; Moreno et al., 1999; Morris et al., 1999; Neumeister et al., 2004). A subgroup of patients suffering from major depression has lower blood tryptophan values and lower levels of 5-hydroxy-indole-aceticacid (5-HIAA) in the cerebrospinal fluid (Asberg et al., 1976; Asberg and Traskman, 1981; Oreland et al., 1981; Traskman et al., 1981). Microdialysis studies in laboratory animals have shown increased extracellular 5-HT levels with tryptophan loading (van der Stelt et al., 2004; Westerink and Devries, 1991). Tryptophan may therefore have some antidepressant potential, and since the late eighties the compound can be obtained over-the-counter as a dietary supplement. However, several cases of eosinophilia-myalgia syndrome have been reported caused by the intake of contaminated tryptophan, which has harmed its image as a relatively safe antidepressant. A recent meta-analysis of clinical trials with tryptophan and 5-hydroxy-tryptophan suggested modest antidepressant efficacy of these serotonin precursors, but their clinical usefulness was questioned since safe and more effective alternatives are available (Shaw et al., 2002).

6 Scope of this thesis

To augment or not to augment, that is the question. The pros and cons of SSRI augmentation strategies have been detailed extensively in **chapter one**. SSRI augmentation strategies have their limitations and they are certainly not without risk, but they may be the only viable option to improve antidepressant treatment in the short term. Convincing clinical evidence in support of SSRI augmentation strategies is very limited, mainly because potent and selective 5-HT receptor antagonists are not yet available for use in humans. However, this does not apply for studies in laboratory animals. The aim of the present thesis is to further explore SSRI augmentation by addressing a number of important questions that can be raised with this approach. For instance, does SSRI augmentation lead to increased neuronal activity in brain areas that have been associated with major depression? Expression of immediate early genes, assessed either via mRNA or corresponding protein, has been used as index for the postsynaptic effects of antidepressants. Although far from being a selective measure for antidepressant effects it may give some idea which neuronal networks in the brain are initially activated by antidepressants. In **chapter two** the expression of the immediate early gene *c-fos* is used to assess the neuronal activation pattern elicited by a single dose of the SSRI citalopram both in absence and presence of the 5-HT_{1A} receptor antagonist WAY 100635. The results are discussed in the context of the available preclinical and clinical literature regarding the brain areas putatively involved in the neurobiology and pharmacotherapy of affective disorders, including major depression.

The concept of SSRI augmentation with a 5-HT_{1A} receptor antagonist is strongly based on Blier's desensitization hypothesis. There is indeed a large body of evidence that supports the desensitization of 5-HT_{1A} autoreceptors following chronic antidepressant treatment. The question is whether this also applies for 5-HT_{1B} autoreceptors and postsynaptic 5-HT_{1A} receptors involved in long loop type of feedback. In **chapter three** intracerebral microdialysis in conscious rats is used to assess the effect of chronic treatment with the SSRI citalopram on the sensitivity of 5-HT_{1B} receptors. Importantly, measurements were performed while the animals were still on the drug to avoid rapid desensitization of 5-HT_{1B} receptors during washout. The effects of chronic SSRI treatment on stress (e.g. HPA-axis activity) are well documented, both in humans and animals. Accordingly, several peripheral stress markers were also measured in the study. Previously, it was demonstrated that postsynaptic 5-HT_{1A} receptors in the amygdala, involved in long loop type of feedback, desensitize following chronic treatment with an SSRI. In **chapter four** it is investigated whether this also applies for such receptors in the prefrontal cortex, an area that has been strongly implicated in major depression.

Another important question concerns the availability of the serotonin precursor tryptophan. Serotonin does not enter the brain, and 5-HT neurons have to synthesize the transmitter from tryptophan, which is actively transported into the brain. It is conceivable that the effect of an SSRI on extracellular 5-HT levels and in particular its augmentation with 5-HT receptor antagonists is restricted by the availability of tryptophan. This question is addressed in **chapter five** using two different approaches to manipulate serotonin synthesis viz. oral tryptophan supplementation and inhibition of serotonin synthesis by retrograde microdialysis of NSD 1015. The latter compound inhibits the enzyme aromatic aminoacid decarboxylase, the enzyme responsible for the conversion of 5-hydroxytryptophan into serotonin. **Chapter six** involves the effect of chronic SSRI treatment on total serotonin content, synthesis and metabolism. Intracellular serotonin stores depend on both synthesis and reuptake of previously released serotonin. It is conceivable that prolonged reuptake inhibition will deplete these stores. Another worrying aspect of chronic antidepressant treatment is the clinical phenomenon called rebound depression. When antidepressant therapy is suddenly discontinued, patients have been reported to relapse into a depressive state, emphasizing the need to slowly phase out SSRI treatment. An analogy may be found with the washout period in preclinical chronic treatment studies, which is commonly used to avoid interference with the pharmacological probes. Arguably, the effects of a sudden discontinuation of treatment are more prominent than the effect of the treatment itself. The latter possibility is also investigated in **chapter six** by comparing the effects of chronic SSRI treatment on total serotonin content, synthesis and metabolism in presence and absence of a washout period.

The relevance of the data presented in this thesis and the possible consequences thereof for future antidepressant research and drug development will be discussed briefly in **chapter seven.**

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CHAPTER 2

Effect of 5-HT augmentation on Fos immunoreactivity

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Abstract

The consequences of pharmacologically evoked augmented serotonin (5-hydroxy tryptamine; 5-HT) release on neuronal activity in the brain, as reflected by the cellular expression of the immediate early gene c-fos, were studied. Wistar rats were treated with saline, the 5-HT-reuptake inhibitor citalopram (10 μmol/kg s.c.), the 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-*N*-(2-pyridyl)cyclohexane carboxamine trihydrochloride (WAY 100635, 1 μmol/kg s.c.), or the combination of both drugs. At the given dosages, the combination of the drugs has previously been shown to enhance the cerebral release of 5-HT. 2 ½ hours after administration the brains were fixated, and Fos protein was histologically stained and quantified. The paraventricular nucleus of the hypothalamus, the central nucleus amygdala, the ventromedial hypothalamic nucleus, the dorsolateral striatum, and the nucleus accumbens shell were particularly responsive to increased 5-HT release. The results, illustrating the synergistic consequence of the combined drug treatments, are discussed in terms of activity of the limbic-hypothalamic-pituitary-adrenocortical system.

1. Introduction

The therapeutic response to selective serotonin reuptake inhibitors is generally attributed to enhanced extracellular levels of 5-HT (5-hydroxy tryptamine) and is usually not seen until after 2-4 weeks of medication. A possible explanation for the delayed onset of action is the gradual desensitisation of inhibitory 5-HT autoreceptors, as proposed by Blier et al. (1987a). Such consequences of desensitisation can be mimicked by blocking the autoreceptors with antagonists, which instantaneously augments the selective serotonin reuptake inhibitor-induced increase in extracellular 5-HT and may thus accelerate the clinical response to the selective serotonin reuptake inhibitor. Indeed, in several animal studies augmentation of 5-HT release was observed following acute co-administration of selective serotonin reuptake inhibitors with a 5-HT_{1A} receptor antagonist (Hjorth, 1993; Romero et al., 1996; Sharp et al., 1997; Cremers et al., 2000b) or with a 5-$HT_{1B/1D}$ receptor antagonist (Rollema et al., 1996; Sharp et al., 1997; Gundlah et al., 1997; Cremers et al., 2000b).

Compounds such as the anorectic drug fenfluramine and several antidepressants including the Selective serotonin reuptake inhibitors, which increase the release of 5-HT, also induce an enhanced expression of c-fos, a marker of neuronal activity, throughout the brain (Richard et al., 1992; Li and Rowland, 1996; Javed et al., 1997; Javed et al., 1998; Veening et al., 1998). Following challenge with a 5-HT_{1A} or 5-$HT_{2A/C}$ receptor agonist, Fos expression was demonstrated in several brain areas, including the prefrontal cortex, central nucleus of the amygdala, striatum, nucleus accumbens, and paraventricular nucleus of the hypothalamus (Compaan et al., 1996; Moorman et al., 1996; Leslie et al., 1993). The consequences of pharmacologically evoked augmented 5-HT release for neuronal activity in the brain, as reflected by the cellular expression of the immediate early gene c-fos, have not yet been reported.

The effect of augmentation of extracellular 5-HT depends on the brain area investigated and the dose of selective serotonin reuptake inhibitor. To show their effect on 5-HT release, autoreceptors need to be sufficiently activated by endogenous 5-HT. Using an optimal dose of both citalopram, a selective serotonin reuptake inhibitor, and the 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)-*N*-(2-pyridyl)cyclohexane carboxamine trihydrochloride (WAY 100635) (Cremers et al., 2001a, b), we studied the effect of combined treatment with citalopram and WAY 100635 on c-fos expression in several brain areas.

2. Materials and methods

2.1. Animals

Male Wistar rats (Harlan, Zeist, the Netherlands) weighing 200-220 g were housed individually under a 12-h light/dark cycle with free access to food and water. The experiments were performed during the light period.

2.2. Design of the study

To minimize the effects of stress, all rats received saline (1 ml/kg/day i.p. and 0.3 ml/kg/day s.c.) for 7 days prior to drug challenge (Sebens et al, 2000). The following day a single dose of saline (1 ml/kg, n=6), citalopram (10 μmol/kg, n=6), WAY 100635 (1 μmol/kg, n=6), or a combination of citalopram (10 μmol/kg, n=6) and WAY 100635 (1 μmol/kg, n=6) was administered s.c. The animals were perfused transcardially under pentobarbital anaesthesia 2.5 h after the final drug injection. The study was approved by the Committee on Animal Bio-ethics of the University of Groningen.

2.3. Drugs

Citalopram hydrobromide (generously supplied by Lundbeck, Denmark, courtesy of Dr. Sanchez) and WAY 100635 (synthesized in our own laboratory, courtesy of Dr. Y. Liao) were dissolved in saline. Substances were injected s.c. in a volume of 1 ml/kg; injection of the solutions did not produce any apparent discomfort.

2.5. Immunohistochemistry

Animals were perfused under deep anaesthesia (pentobarbital 100 mg/kg) with saline for 1 min followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 for 15 min. Brains were removed and postfixed overnight at 4 ^{o}C in 4% paraformaldehyde solution before being stored in 50 mM Tris-buffered saline pH 7.4 containing 0.1% Na-azide. After cryoprotection by overnight immersion in a buffered (50 mM Tris/HCl buffer pH 7.4) 30% sucrose solution at room temperature, the brains were sliced into 30-μm coronal sections using a cryostat microtome. Immunostaining was performed on free-floating sections, according to the previously described procedure (Sebens et al., 1995). Briefly, sections were pretreated with 0.3% H_2O_2 and preincubated in 4% normal goat serum before the Fos primary antiserum (1:10.000, Oncogene Science, Ab-5, Cambridge, MA, USA) was added. A biotinylated anti-rabbit secondary antibody (1:800, Vector Laboratories, Burlingame, CA, USA) was used, followed by an avidin-

biotinylated horseradish peroxidase complex (1:125, Vector Laboratories, Burlingame, CA, USA). Intermittent washing was done with Tris-buffered saline. The peroxidase reaction was developed with DAB (3,3' diaminobenzidine)-Ni (ammoniumnickelsulphate) / H_2O_2 in Tris buffer. To control for the specificity of immunoreactivity, some of the sections were incubated with omission of the primary or the secondary antibody.

2.6. Quantification and statistical analysis

Schematic drawings of the representative sections used for counting c-Fos-positive cells are shown in Fig. 1. The areas counted are indicated by grey-filled squares. Fos-positive cells were counted using a computerized image analysis system. The selected area from structures of interest was digitized using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMBR microscope (LEICA, Wetzlar, Germany) at x10 magnification. The numbers of Fos-positive nuclei were counted using a computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). The resulting data are reported as the number of positive cells/0.13 mm^2 (Sebens et al, 1995, Sebens et al, 2000).

Fos-positive cells were counted bilaterally and averaged per animal. Per experimental group the mean number (± S.E.M.) of Fos-positive cells was determined. The data of the various groups were compared using a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls or the Dunn's test for multiple comparison procedures. The differences were considered significant if $P < 0.05$.

3. Results

Two hours and 30 minutes after administration of either saline, citalopram or WAY 100635, Fos immunoreactivity was seen throughout the whole brain. The selective serotonin reuptake inhibitor citalopram caused a specific regional pattern of Fos immunoreactivity. Representative sections are shown in Fig. 2. The distribution of Fos-positive cells is shown in Fig. 3. Compared to the c-fos response following saline administration, the most prominent effects of citalopram challenge were seen in the prefrontal cortex, central nucleus of the amygdala, supraoptic nucleus and the paraventricular nucleus of the thalamus, while no effect was seen in the lateral septum, dorsomedial striatum, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus and the dorsal raphe nucleus (Fig.1).

Compared to saline, the 5-HT_{1A} receptor antagonist WAY 100635 increased the number of c-fos positive cells in the prefrontal cortex, nucleus accumbens shell and core, dorsomedial striatum and dorsal raphe nucleus. In no other areas were significant net effects of WAY 100635 seen.

Coadministration of citalopram and WAY 100635 resulted in a clear increase of Fos-positive cells in all regions of interest, apart from the lateral septum. Four different patterns of response to combined treatment were distinguished: firstly, no difference compared to either one of the treatments (lateral septum); secondly, an increase equivalent to the effect seen following acute citalopram injection (prefrontal cortex, supraoptic nucleus and paraventricular nucleus of the thalamus); thirdly, an increase comparable to the effect of WAY 100635 (nucleus accumbens core, dorsomedial striatum, and dorsal raphe nucleus and dorsomedial hypothalamic nucleus); and fourthly, an augmentation of the effect of one of the drugs (nucleus accumbens shell, dorsolateral striatum, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus and central nucleus of the amygdala).

Brain areas in which no Fos immunoreactivity was found in both control and any of the treated animals included the median raphe nucleus and the hippocampus.

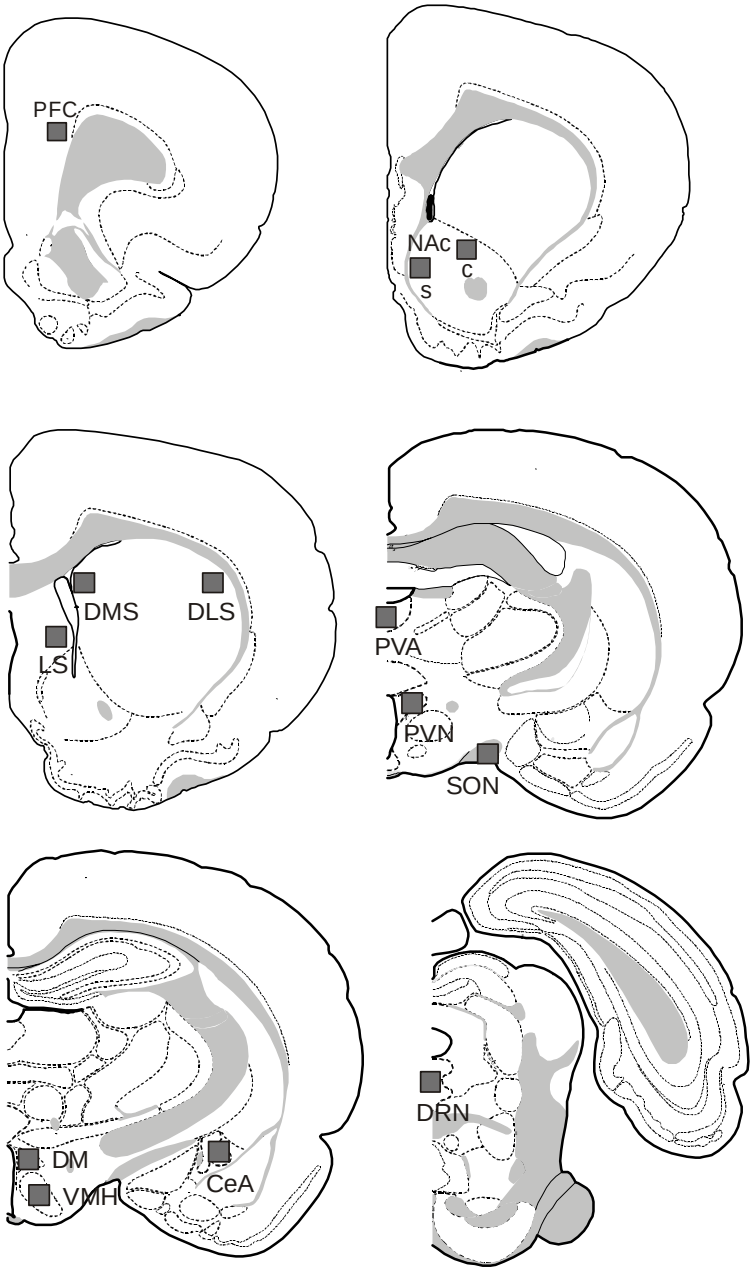


Fig. 1. *Schematic representation of the areas used for quantification of Fos protein-positive cells. Grey-filled squares indicate the regions counted. PFC, prefrontal cortex; NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; LS, lateral septum; DMStr, dorsomedial striatum; DLStr, dorsolateral striatum; PVN, paraventricular nucleus; SON, supraoptic nucleus; CeA, central nucleus of the amygdala; PVA, paraventricular nucleus; DRN, dorsal raphe nucleus; VMH ventromedial hypothalamic nucleus, DM dorsomedial hypothalamic nucleus.*

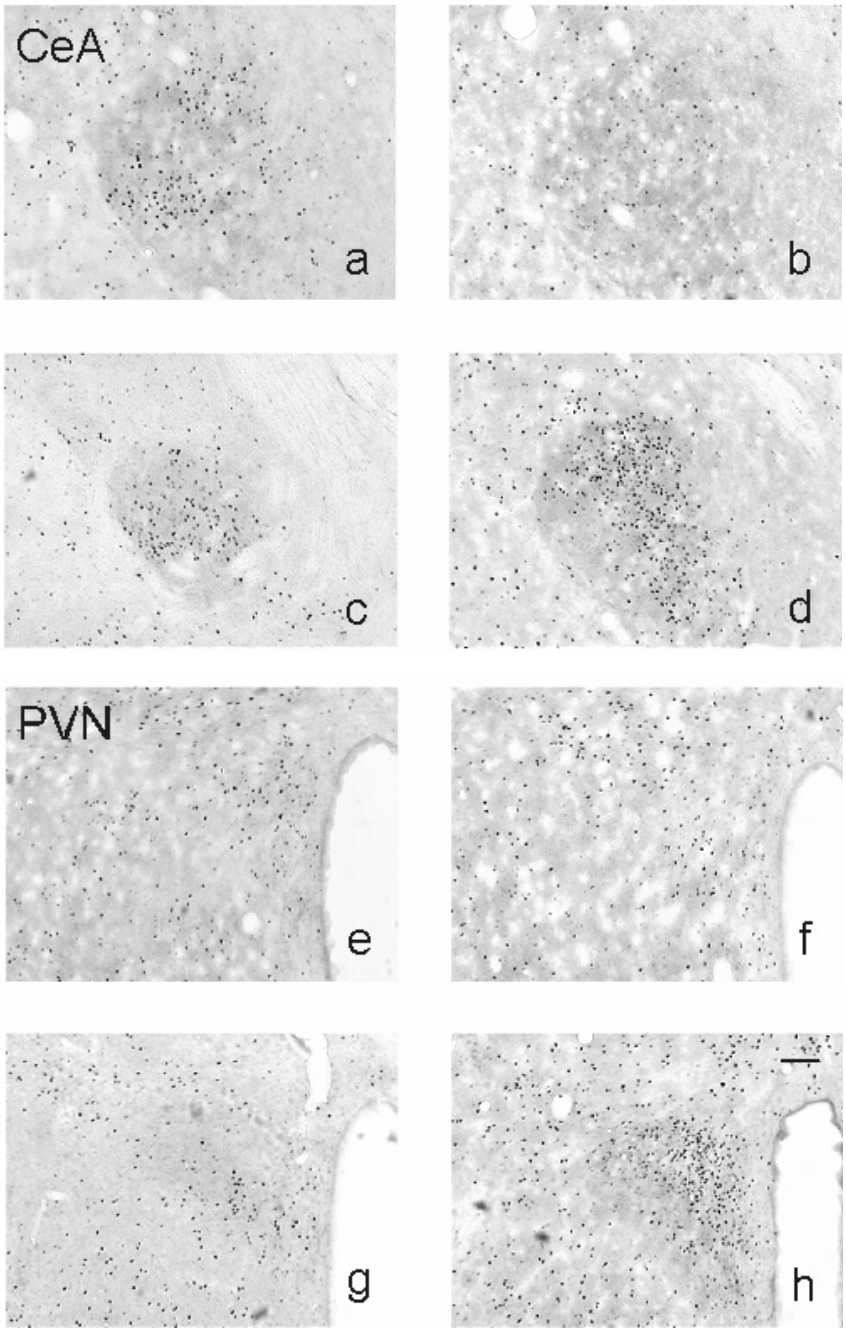
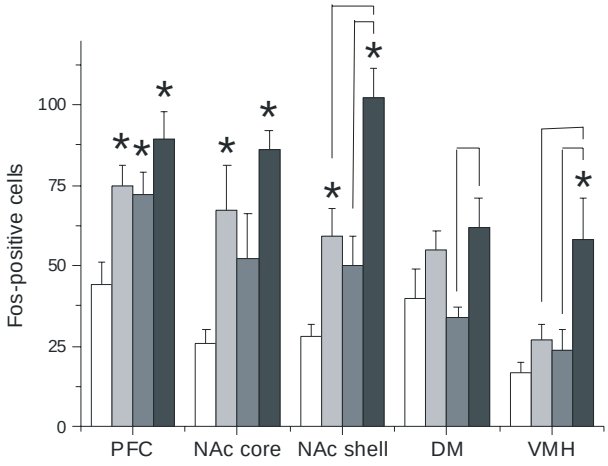
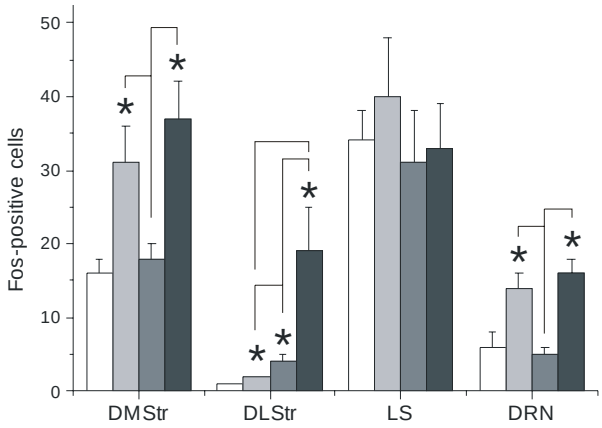


Fig. 2. *Representative samples of the distribution of Fos immunoreactivity following saline, WAY 100635, and the combination of WAY 100635 and citalopram, respectively, in the central nucleus of the amygdala (a, b, c and d) and in the paraventricular nucleus of the hypothalamus (e, f, g and h). Scale bar = 100 μm, CeA = central nucleus of the amygdala, PVN = paraventricular nucleus of the hypothalamus.*


Fos-positive cells
0
25
50
75
100
PFC
NAc core
NAc shell
DM
VMH


Fos-positive cells
0
10
20
30
40
50
DMStr
DLStr
LS
DRN

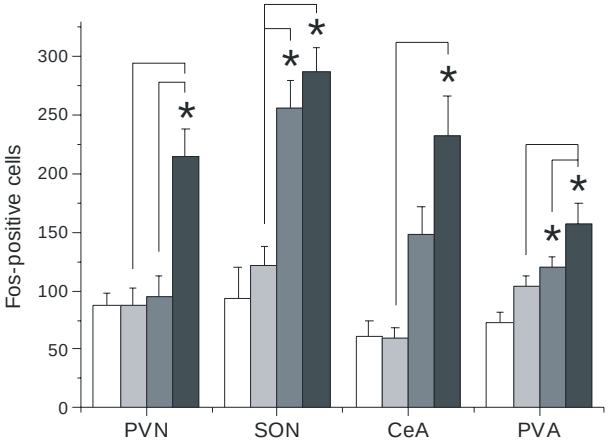

Fos-positive cells
0
50
100
150
200
250
300
PVN
SON
CeA
PVA

Fig. 3. *Distribution patterns of Fos protein-positive cells (mean number ± S.E.M.) induced by a challenge dose of saline (open bars), WAY 100635 (light grey filled bars), citalopram (grey filled bars), or a combination of citalopram and WAY 100635 (dark grey filled bars). Brain regions investigated were: PFC, prefrontal cortex; NAc shell, nucleus accumbens shell; NAc core, nucleus accumbens core; LS, lateral septum; DMStr, dorsomedial striatum; DLStr, dorsolateral striatum; PVN, paraventricular nucleus; SON, supraoptic nucleus; CeA, central nucleus of the amygdala; PVA, paraventricular nucleus; DRN, dorsal raphe nucleus; VMH ventromedial hypothalamic nucleus, DM dorsomedial hypothalamic nucleus. * Significantly different (P < 0.05, Student's t-test) from Sal. Connecting lines indicate a significant difference between treatment groups as depicted in graph (P < 0.05, Student's t-test).*

4. Discussion

Compared to saline treatment, co-administration of the selective serotonin reuptake inhibitor citalopram and 5-HT_{1A} receptor antagonist WAY 100635 resulted in a significant increase in c-fos-positive cells in all the investigated brain regions, except the lateral septum. In the prefrontal cortex, supraoptic nucleus and paraventricular nucleus of the thalamus, this increase can be explained as the exclusive action of citalopram, whereas in the nucleus accumbens core, dorsomedial striatum, dorsomedial hypothalamic nucleus and dorsal raphe nucleus the induction of c-fos appeared to be mainly, if not exclusively, mediated by WAY 100635. Using a dose of citalopram previously shown to activate 5-HT_{1A} receptors (Cremers et al., 2000b) we observed an additive or more than additive effect (augmentation) of the compounds in the central nucleus of the amygdala, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, nucleus accumbens shell and dorsolateral striatum.

Fenfluramine (Richard et al., 1992; Li and Rowland, 1996; Javed et al, 1997; Javed et al 1998) and other compounds affecting the release of 5-HT (e.g. methylenedioxymethamphetamine, chloroamphetamine and the monoamine oxidase inhibitor tranylcypromine; Moorman et al., 1995; Moorman and Leslie, 1996, Stephenson et al., 1999) induce c-fos expression in the forebrain (prefrontal cortex, nucleus accumbens, lateral septum, caudate putamen, bed nucleus of the stria terminalis), the midbrain (paraventricular nucleus of the hypothalamus, paraventricular nucleus of the thalamus, central nucleus of the amygdala) and brainstem (lateral parabrachial nucleus, nucleus of the solitary tract).

Selective serotonin reuptake inhibitors induce c-fos activation in the central nucleus of the amygdala, bed nucleus of the stria terminalis and lateral parabrachial nucleus (Veening et al., 1998). Taken together with the present observations concerning the prefrontal cortex, nucleus accumbens and paraventricular nucleus of the thalamus, the pattern is similar to that seen following activation with fenfluramine, indicating that 5-HT plays a specific role in the regulation of c-fos expression.

Receptors that are thought to induce c-fos expression include those that stimulate the inositol phosphate pathway and those that increase intracellular cAMP or Ca^{2+} concentration (Sheng et al., 1990a and b, 1991, for review see Hughes and Dragunow, 1995; Chen et al., 1999). Most serotonergic receptors are positively coupled to their second messenger systems via G_s protein (5-HT_4, 5-HT_6 and 5-HT_7), G_q/G_{11} (5-HT_2) or via ion channels (5-HT_3) (for review see Uphouse, 1997). Stimulation of the Gi/Go protein-coupled 5-HT_1 receptor family (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D}), however, induces inhibition of cAMP and may hence inhibit c-Fos expression. If the effect of selective serotonin reuptake inhibitors on c-fos is indeed directly mediated by an

increase in serotonin, the pattern of Fos immunoreactivity is the result of an interplay of all positively coupled serotonergic receptors. Furthermore, blockade of the inhibitory 5-HT_{1A} receptor should increase serotonergic activity, especially in brain areas heavily controlled by 5-HT_{1A} receptors. Although we observed an augmentation of the effect of selective serotonine reuptake inhibitors in the nucleus accumbens shell, dorsolateral striatum, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus and central nucleus of the amygdala, none of these regions have a high density of 5-HT_{1A} receptors or are heavily controlled by raphe 5-HT_{1A} receptors (Pazos and Palacios, 1985 b; Steinbush and Nieuwenhuys, 1981). Apart from the low or absent 5-HT_{1A} receptor density, autoreceptor inhibition does not explain the observed results because, in contrast with microdialysis results, no augmented c-fos response could be observed in serotonergic brain areas such as the dorsal raphe nucleus, median raphe nucleus, prefrontal cortex or hippocampus. These seeming discrepancies are in agreement with results reported in previous studies and have puzzled their authors (Compaan et al., 1996, Hajós-Korcsok and Sharp, 1999).

The pattern in the rat brain of c-Fos expression in the rat brain following the administration of citalopram corresponds neither with the distribution of a particular 5-HT receptor type (Pazos and Palacios, 1985a and b; Morilak et al., 1993, Ward et al., 1995; Kilpatrick et al., 1987) nor with the density of 5-HT containing nerve terminals (Steinbush and Nieuwenhuys, 1981). This mismatch between receptors, 5-HT innervation and Fos expression does not point to direct coupling between any of the 5-HT receptors and c-fos, but rather indicates that c-fos expression is mediated indirectly through other pathways and/ or receptors.

This contention is supported by the notion that stimulation of the 5-HT_{1A} receptor induces Fos immunoreactivity despite its negative coupling to its transducing proteins. So, if c-fos expression is mediated directly via the 5-HT_{1A} receptor, this would more likely lead to an inhibition of its expression (Compaan et al., 1996, Hajós-Korcsok, 1999).

Blocking the 5-HT_{1A} receptor induced an increase in Fos expression in the cortex, nucleus accumbens and striatum. Javed et al. (1998) found the same trend in the cortex. Such differential patterns of Fos expression following administration of the 5-HT_{1A} receptor antagonist WAY 100635 may not only be attributed to brain region specificity but also to the physiological state of the animals (e.g. stress exposure).

We observed augmentation of citalopram-induced Fos expression by WAY 100635, which is in contrast with Javed et al. (1998), who used a combination of WAY 100635 with fenfluramine. Fenfluramine, however, in contrast with citalopram, releases 5-HT via a non-exocytotic mechanism which is independent of the firing rate and hence of 5-HT_{1A} autoreceptor regulation.

The most consistent increases in Fos expression in the augmentation paradigm were observed in the paraventricular nucleus of the hypothalamus, ventromedial nucleus of the hypothalamus, central nucleus of the amygdala, nucleus accumbens shell and the dorsolateral striatum: the first four areas belong to the limbic system. Although no 5-HT_{1A} receptors are present in the paraventricular nucleus of the hypothalamus, co-administration of citalopram with an antagonist seems to be essential to induce elevation of Fos expression in this area. Most likely, corticotropin-releasing hormone cells of the paraventricular nucleus of the hypothalamus are indirectly activated by innervations from other 5-HT_{1A} receptor-containing regions. Indeed, both the bed nucleus of the stria terminalis and the central nucleus of the amygdala innervate the paraventricular nucleus of the hypothalamus (Gray et al., 1989), and involvement in the regulation of the hypothalamic-pituitary-adrenocortical axis has been demonstrated (Feldman et al., 1990, 1994). In addition, the observation that lesion of the ventromedial hypothalamic nucleus disrupts hypothalamic-pituitary-adrenocortical axis feedback control indicates its (in)direct influence on other brain areas of the hypothalamic-pituitary-adrenocortical system (Suemaru et al., 1995). It is remarkable that, when using c-fos as a marker, especially those areas belonging to the limbic-hypothalamic-pituitary-adrenocortical axis responded to serotonergic augmentation.

Dysregulation of the limbic-hypothalamic-pituitary-adrenocortical axis has been considered as part of the pathophysiology of both depression and anxiety. Activity of the paraventricular nucleus of the hypothalamus induces an increase in plasma levels of the stress hormone cortisol (corticosterone in rats) through the release of adrenocorticotropin from the pituitary, which is controlled by the release of corticotropin-releasing hormone from the paraventricular nucleus of the hypothalamus. It has been hypothesized by Barden et al (1995, for review see Holsboer, 1996) that the mood stabilizing effect of antidepressants is achieved by their action on the limbic-hypothalamic-pituitary-adrenocortical system. This proposal is not only in line with the present findings, but it also emphasizes the clinical relevance of our observations. Measuring plasma cortisol in depressed or anxious patients gives an indication of the functioning of the paraventricular nucleus of the hypothalamus and the limbic-hypothalamic-pituitary-adrenocortical system. Depressed patients exhibit a blunted response to activation of the hypothalamic-pituitary-adrenocortical axis, which is restored by therapy with antidepressants. Probably an altered functionality of the 5-HT_{1A} receptor contributes to this effect. It would therefore be interesting to follow patients chronically treated with antidepressants, simply by measuring the cortisol release after challenge with a 5-HT_{1A} agonist.

In conclusion, with c-fos expression as a marker of neuronal activity, the greatest effects were seen in areas belonging to the limbic-hypothalamic-pituitary-adrenocortical system, indicating

that the hypothalamic-pituitary-adrenocortical axis is a potential target for selective serotonin reuptake inhibitors. These augmentation effects do not necessarily correspond to the effects on the release of 5-HT. Whereas measuring extracellular 5-HT concentration provides insight into processes directly controlling the release of 5-HT, c-fos expression may provide information about which brain regions are activated as an indirect consequence of the manipulation of the release and activity of 5-HT neurons.

In some of our previous studies it was shown that repeated activation of the c-fos system leads to a rapid attenuation (tolerance) of drug-evoked responses (Sebens et al., 1995, 1996). Whether such c-fos desensitisation can be found following chronic treatment with selective serotonin reuptake inhibitors in the limbic-hypothalamic-pituitary-adrenocortical system has as yet to be assessed.

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CHAPTER 3

The effect of chronic selective serotonin reuptake inhibitor treatment on serotonin$_{1B}$ receptor sensitivity and HPA-axis activity

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Abstract

The authors have investigated 5-HT_{1B} receptor function in prefrontal cortex and dorsal hippocampus as well as the HPA-axis response after subchronic (24 hr) and chronic (15 day) treatment with the SSRI citalopram. All experiments were carried out in presence of citalopram to prevent rapid resensitisation of the 5-HT_{1B} receptors. Moreover, this more closely resembles the clinical situation. The concentration of citalopram was measured in both brain areas to ensure comparable levels in the different treatment groups. Using microdialysis, the authors found that under those conditions the effect of the 5-HT_{1B} receptor antagonists SB 224289 and the mixed 5-$HT_{1B/1D}$ receptor antagonist GR 127935 on extracellular levels of 5-HT was unaltered by duration of treatment. Basal levels of 5-HT however, were increased in the dorsal hippocampus following chronic treatment. In addition, plasma levels of the catecholamines adrenaline and noradrenaline and the HPA-axis hormones ACTH and corticosterone were all decreased after chronic treatment.

1. Introduction

Antidepressants are clinically effective only after prolonged treatment, indicating that adaptive mechanisms are involved in the therapeutic effect. This delayed response may be linked to a gradual desensitization of firing rate and release controlling 5-HT autoreceptors (Blier et al., 1987). This idea was based on electrophysiological experiments in rats, wherein acute administration of antidepressants decreased the firing rate of 5-HT neurons, which normalized after prolonged administration (Blier et al., 1987; Chaput et al., 1986).

Using 5-HT$_{1A}$ or 5-HT$_{1B}$ receptor antagonists, microdialysis studies in rodents have demonstrated that acute administration of an SSRI activates both types of autoreceptors (Cremers et al., 2000a; Hjorth, 1993; Invernizzi et al., 1997; Rollema et al., 1996). Both electrophysiology and microdialysis have revealed a reduction of 5-HT$_{1A}$ receptor functionality following chronic administration (Cremers et al., 2000b; Invernizzi et al., 1994; Kreiss and Lucki, 1995; Le Poul et al., 1995). In contrast with single unit recordings, changes in 5-HT$_{1B}$ receptor functionality following chronic antidepressant treatment could not be demonstrated using microdialyis (Auerbach and Hjorth, 1995; Bosker et al., 1995; Chaput et al., 1986; Cremers et al., 2000b; Davidson and Stamford, 1997; Moret and Briley, 1996).

Levels of 5-HT$_{1B}$ mRNA on the other hand, are decreased following chronic treatment with antidepressants, but rapidly return to normal after discontinuation of the antidepressant (Anthony et al., 2000; Neumaier et al., 1996). Arguably, a loss of presynaptic 5-HT$_{1B}$ receptor function may be retrieved within a few days after discontinuation of the drug. Hence, the possibility that presynaptic 5-HT$_{1B}$ receptors rapidly resensitize is worth investigating. This is further emphasized by the common practice in microdialysis studies to use a washout period (2-7 days) to minimize pharmacological interference by residual antidepressant.

Next to these central serotonergic adaptations, peripheral alterations of stress hormone release might also play a role in the therapeutic effect of long term treatment with SSRIs. Stress activates many physiological systems, such as the sympathetic system, resulting in rapid release of the catecholamines adrenaline and noradrenaline from the adrenal medulla. This is followed by activation of the hypothalamic-pituitary-adrenal (HPA)-axis, resulting in release of ACTH from the pituitary which induces the release of cortisol (corticosterone in rats) from the adrenal cortex. Prolonged elevation of catecholamines and cortisol by chronic stress could be a factor in stress related pathology, including depression. Pharmacological intervention in stress related processes might therefore help to slow down exacerbation of depressive symptoms. This is supported by the observation that the HPA-axis hyperactivity in depressed patients is normalized after clinical

remission due to chronic antidepressant treatment (Barden et al., 1995; Holsboer and Barden, 1996).

In this study, the authors have investigated whether presynaptic 5-HT_{1B} receptors become less responsive during chronic treatment with the SSRI citalopram. Citalopram was administered via osmotic minipumps to obtain steady state levels within the clinically effective range (Cremers et al., 2000b). To circumvent resensitization of presynaptic 5-HT_{1B} receptors, all microdialysis experiments were performed in the presence of the drug. Extracellular levels of 5-HT and citalopram were measured in prefrontal cortex and dorsal hippocampus, brain areas that have been implicated in depression and anxiety, respectively. Responsiveness of presynaptic 5-HT_{1B} receptors was measured by the ability of the 5-HT_{1B} antagonists GR 127935 and SB 224289 to augment the effect of citalopram. In addition, the authors have measured plasma levels of catecholamines, ACTH and corticosterone to investigate the effect of treatment duration on peripheral levels of stress hormones.

2. Materials and methods

2.1 Animals

Male Harlan rats (Zeist, Netherlands) weighing 285-320 g were housed eight per cage under standard conditions (22-24 ^{o}C, 12/12 light/dark cycle, food and water ad libitum). Following implantation of the minipump, rats were housed in pairs. After stereotaxic surgery and during the microdialysis experiments the rats were housed separately. All animal experiments were according to the governmental guidelines for care and use of laboratory animals and were approved by the Committee for Animal Research of the Medical Faculty of the Groningen University.

2.2 Drugs

The following drugs were used: Citalopram hydrobromide (kindly donated by Lundbeck (Denmark) courtesy Dr. Sanchez), GR 127935 (N-[4-methoxy-3-(4-methyl-1-piperizinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazole-3-yl)[1,1'-biphenyl]-carboxamide, synthesised in our own laboratory, courtesy Dr. Y Liao and Dr. M Mensonides) and SB 224289 (2,3,6,7-tetrahydro-1'-methyl-5-(2'-methyl-4'-[(5-methyl-1,2,4-oxqadiazole-3-yl)-biphenyl-4-yl]carbon-yl)furo[2,3-f]indole-3-spiro-4'-piperidine oxalate, purchased from Sigma-Alldrich). GR 127935 was dissolved in saline with a drop of acetic acid and administered at a dose of 1 μmol/kg, SB 224289 was dissolved in a 10% dimethylsulfoxide (DMSO)-saline solution and administered at a dose of 4 mg/kg. Both substances were injected subcutaneously in a volume of 1 ml per kg. Both dosages were chosen for their ability to augment SSRI induced increase of 5-HT to the same extent (Cremers et al., 2000a; Roberts et al., 1999).

2.3 Surgery

Minipumps:

Osmotic minipumps (2ML2 Alzet, USA, 5 μl/h, 2 weeks) were either filled with saline or 50 mg/ml citalopram hydrobromide dissolved in saline under aseptic conditions. During isoflurane anaesthesia (2.5%, 400ml/min N_2O, 600 ml/min O_2), minipumps were implanted subcutaneously on the left side of the back of the rat.

After 14 days, all minipumps were replaced with citalopram filled minipumps. Hereafter the microdialysis probes were implanted.

Probes:

During isoflurane anaesthesia (2.5 %, 400 ml/min N_2O, 600 ml/min O_2), a home made concentric microdialysis probe (i.d. 220 μm, o.d. 310 μm, AN 69, Hospal, Italy), made of polyacrylonitrile / sodium methyl sulphonate copolymer dialysis fiber was stereotaxically implanted in the prefrontal cortex (PFC) or the dorsal hippocampus (dHC) using the following coordinates: PFC; inscisorbar at -3.3 mm (posterior: +3.5 mm, lateral: +0.9 mm, ventral from dura: –6.0 mm), exposed tiplength was 4 mm. DHC; incisorbar at +5.0 mm (posterior: -4.0 mm, lateral: -1.2 mm, ventral from skull: -5.5 mm), exposed tiplength was 2 mm. (Paxinos and Watson, 1982). The probes were secured in place with dental cement.

2.4 Microdialysis experiments

Microdialysis experiments were performed 24 hrs and 48 hrs after stereotaxic surgery. The saline pretreated animals had hence received a 24 hr citalopram treatment at the start of the microdialysis experiments, which is referred to as subchronic treatment. The citalopram pretreated animals received a 15 day treatment which is referred to as chronic treatment. All experiments were carried out in presence of citalopram delivered systemically via minipumps. To avoid carry over effects, if any, pharmacological challenges with SB 224289 or GR 127935 were randomly allocated for each experiment to the first or second day. Thus, all animals received two different challenges in a randomized fashion.

The probes were perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, pH6-7), using a CMA /102 microdialysis pump at a constant flow rate of 1.5 μl/min.

After a stabilization period of two hours, 15 min samples were collected into vials containing 7.5 μl of 0.02 M acetic acid. All experiments were performed in conscious and freely moving animals.

2.5 Analytical procedures

2.5.1. Serotonin

Analysis of 5-HT was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 μl samples were injected into a HPLC (Shimadzu, LC-10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3 μm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector (ANTEC Leyden, Leiden, the Netherlands) at a potential setting of 500 mV vs. Ag/AgCl

reference electrode. Chromatography was performed at 30 ^{o}C using the integrated column oven of the Antec potentiostat.

The mobile phase consisted of 5 g/l $(NH_4)_2SO_4$, 50 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 4.5% methanol, 30 µl/l triethylamine, adjusted to pH 4.65 with diluted acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.2 fmol/sample (signal to noise ratio 2)

2.5.2 Citalopram

Citalopram was measured according to Oyehaug et al. (1982) with minor modifications. Dialysate samples were injected into an HPLC (1084B Liquid Chromatograph, Hewlett Packard) which was connected with a fluorescence detector (470 Scanning Fluorescence detector, Waters, England) operating at an absorption wavelength of 240 nm, an emission wavelenght of 296 nm, and a slitwidth of 12 nm. Separation was performed using a Supelcosil HPLC column (5 µm, C18, 250 x 46 mm, Supelco, the Netherlands), at ambient temperature. The mobile phase consisted of 46% v/v acetonitrile, 54% v/v potassium dihydrogen phosphate buffer (4.3 g/l) and 1 mM tetramethylammonium, at pH 3.0. The flow rate was set at 0.75 ml/min. The detection limit was 5 nM (signal to noise ratio = 2)

2.5.3.Catecholamines

Norepinephrine and epinephrine were extracted according to Smedes et al. (1982).

Analysis was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 µl samples were injected into an HPLC (Shimadzu, LC-10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3 µm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector (ANTEC Leyden, Leiden, the Netherlands) at a potential setting of 500 mV vs. Ag/AgCl reference electrode. Chromatography was performed at 30^{o} C using the integrated column oven of the Antec potentiostate.

The mobile phase consisted of 0.05 g/l EDTA, 4.1 g/l sodium actetate, 0.14 g/l octane sulphonic acid and 1.8% v/v methanol, at pH 4.10. Flow rate was set at 0.25 ml/min and the detection limit was 0.5 fmol/sample (signal to noise ratio = 3).

2.5.4. Corticosterone

Plasma was extracted twice with diethyl ether, the combined ether layers were evaporated under a stream of nitrogen. The residue was dissolved in mobile phase, vortexed and centrifuged. 50 µl of the supernatant was injected into the HPLC system, consisting of a 1084B Liquid Chromatograph, Hewlett Packard HPLC, connected with a Jasco FP 1520 UV detector. Separation was performed using a Supelcosil HPLC column (5 µm, C18, 250 x 46 mm, Supelco, the Netherlands). Wavelenght was set at 254 nm. The mobile phase consisted of 30 % v/v

acetonitrile and the flow rate was set at 1 ml/min. The detection limit was 0.7 pmol/sample (signal to noise ratio = 3).

2.5.5 ACTH

ACTH levels in plasma were determined using a commercially available RIA kit from Nichols Institute Diagnostics, San Clemente, CA.

2.6 Histology

Following the termination of each experiment, rats were brought under isoflorane aneasthesia in a small plexiglass box. Blood was collected by cardiac puncture during isoflurane anaesthesia (2.5%, 400 ml/min N_2O, 600 ml/min O_2). The animals were killed by decapitation, and the brains were removed and fixed in a 5% formaldehyde solution. Correct placement of the implanted canullas was histologically verified.

2.7 Statistics

The data are presented as percentages of basal values calculated as individuals means of the first four consecutive microdialysis samples. Statistical analysis was performed using Statistica for windows. Treatment effects were compared using a student's T-test or one way ANOVA for repeated measurements. Level of significance was set at $P < 0.05$.

3. Results

3.1. Citalopram brain levels

The extent of augmentation by 5-HT$_{1B}$ receptor antagonists will likely depend on the concentration of the SSRI. To compare the effect of different treatment conditions, the concentrations of the SSRI in the region of interest should at least be comparable. To ensure this, citalopram was measured in dialysates from prefrontal cortex and hippocampus. Citalopram levels in dialysates from prefrontal cortex obtained from subchronically treated animals 17.6 ± 1.5 nM (n = 13) were not significantly different from those obtained from chronically treated animals 13.6 ± 1.2 nM (n = 12).

The same outcome was obtained for dialysates from dorsal hippocampus, showing citalopram levels of 14.2 ± 1.6 nM (n = 13) and 11.7 ± 1.5 nM (n = 12) for sub-chronically and chronically treated animals, respectively. These values are not corrected for in-vitro recovery (~ 25%), suggesting that the actual brain concentrations are considerably higher.

3.2. Basal 5-HT levels in prefrontal cortex and dorsal hippocampus

Cortical 5-HT level in the subchronic treatment group was 22.9 ± 3.6 fmol/sample (n = 13), which was not significantly different from the basal level of 29.6 ± 3.1 fmol/sample (n = 12) as measured in the chronic treatment group.

In dorsal hippocampus basal 5-HT levels in the chronic treatment group (37.0 ± 5.9 fmol/sample (n = 12)), were significantly larger (P = 0.013, Fig. 1) than those measured in the subchronic treatment group (20.5 ± 4.7 fmol/sample (n = 13)).

3.3. GR challenge

Subcutaneous administration of GR 127935 (1 μmol/kg) significantly elevated baseline levels of 5-HT in the prefrontal cortex ($F(10,120) = 2.566$), which did not differ between treatment groups ($F(1,12) = 1.925$ (Fig. 2a).

Levels in the dorsal hippocampus significantly increased to about 120% of baseline value following GR challenge ($F(10,110) = 2.703$), this effect was unaltered by pretreatment ($F(1,11) = 0.155$ n.s.) (Fig. 2b).

3.4. SB challenge

Administration of SB 224289 (8.45 μmol/kg s.c.) significantly augmented 5-HT to about 150% and 180% of baseline value in cortex ($F(10,90) = 8.092$) and dorsal hippocampus ($F(10,80) =$

6.973), respectively (Figs. 3a and b). Statistical analysis did not reveal significant differences between the two treatment groups for both the PFC ($F(1,9) = 2.353$) and dorsal hippocampus ($F(1,8) = 1.386$).

3.5. Corticosterone and ACTH plasma levels

Plasma levels of corticosterone were 0.66 ± 0.05 μM and 0.46 ± 0.07 μM for the subchronic and chronic treatment group, respectively. ACTH levels were 0.13 ± 0.02 nM in the subchronically treated animals and 0.07 ± 0.01 nM in the chronically treated animals. Compared to subchronic treatment, chronic treatment significantly decreased both the plasma levels of corticosterone ($P = 0.048$) and ACTH ($P = 0.031$)(Fig. 4a).

3.6. Noradrenaline and adrenaline plasma levels

Plasma levels of noradrenaline and adrenaline were 11.8 ± 1.4 nM and 14.3 ± 1.8 nM, respectively, for the subchronic treatment group, and 5.5 ± 1.0 nM and 5.7 ± 2.0 nM, respectively, for the chronic treatment group. Compared to subchronic treatment, chronic treatment significantly decreased both the plasma levels of noradrenaline ($P = 0.003$) and adrenaline ($P = 0.008$)(Fig. 4b).

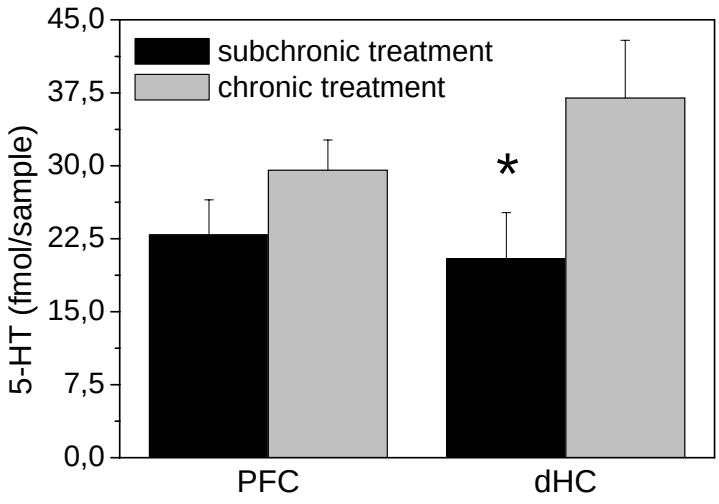

Fig. 1. *Effect of duration of treatment on basal levels of 5-HT in the PFC and dHC. Subchronic (24h) treated animals black bars (n = 13); Chronic treated animals light gray bars (n = 12). * P < 0.02. All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.*

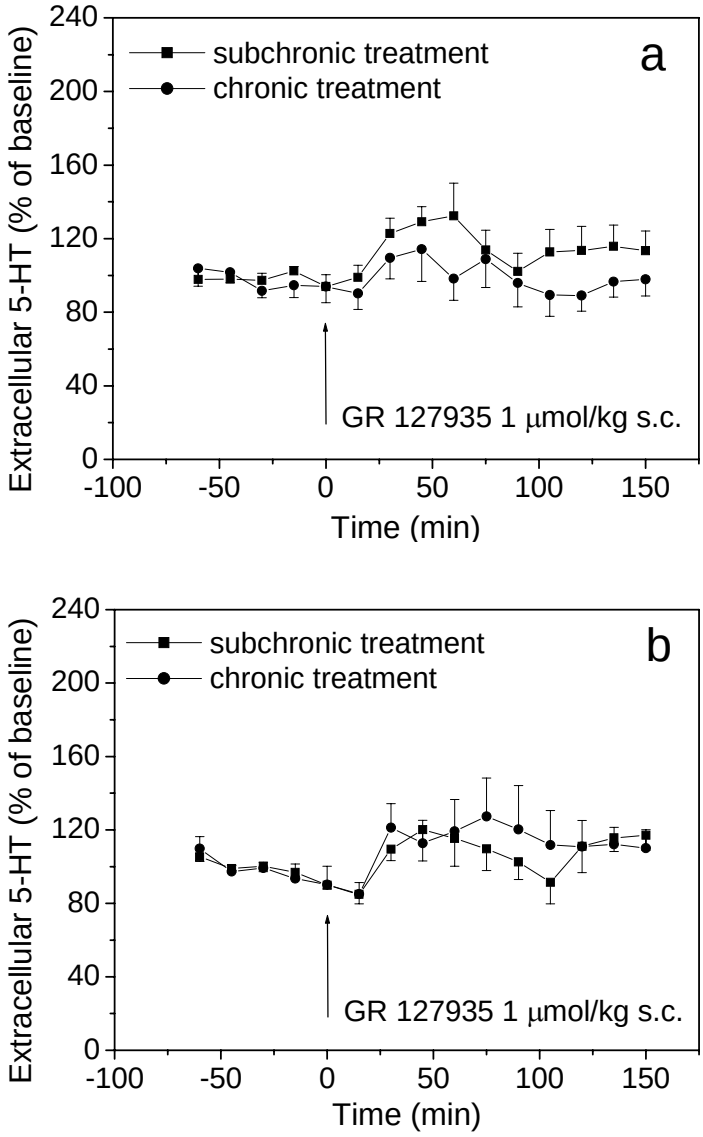

Fig. 2a and b. *Effect of GR 127935 1 μmol/kg s.c. on 5-HT release in PFC (a) and dHC (b). Subchronic treated (n = 8; filled squares); chronic treated (n = 6; filled circles). All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.*

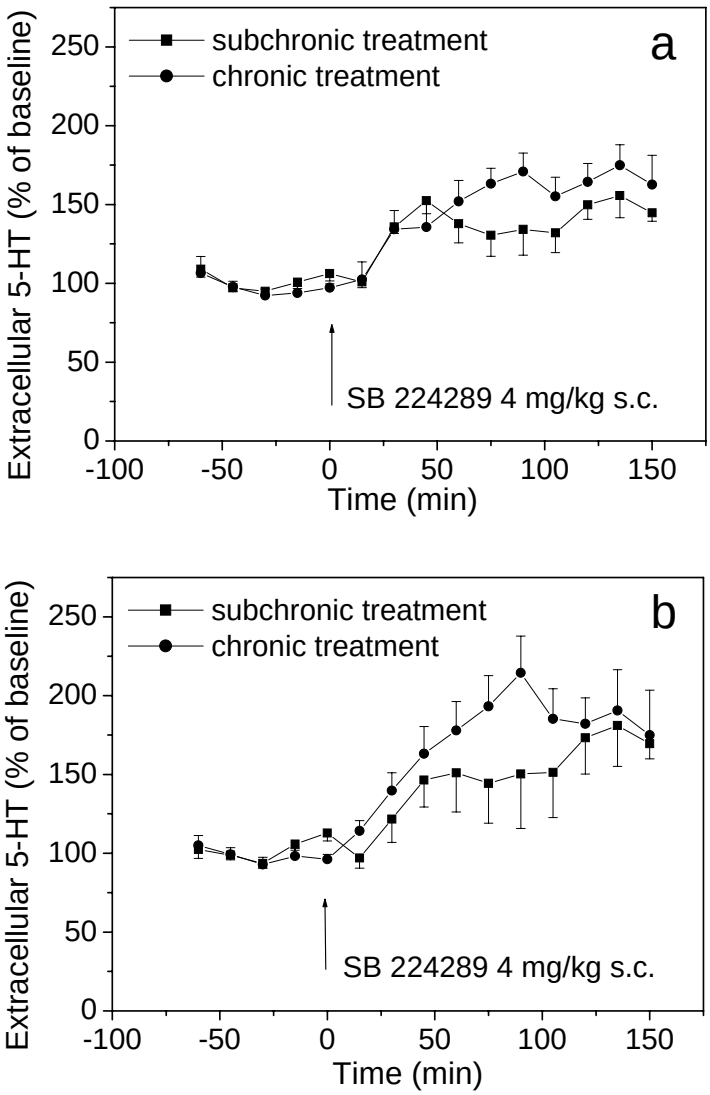

***Fig. 3a and b**. Effect of SB 224289 8.45 µmol/kg s.c. on 5-HT release in PFC (a) and dHC (b). Subchronic treated (n = 8; filled squares); chronic treated (n = 6; filled circles). All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.*

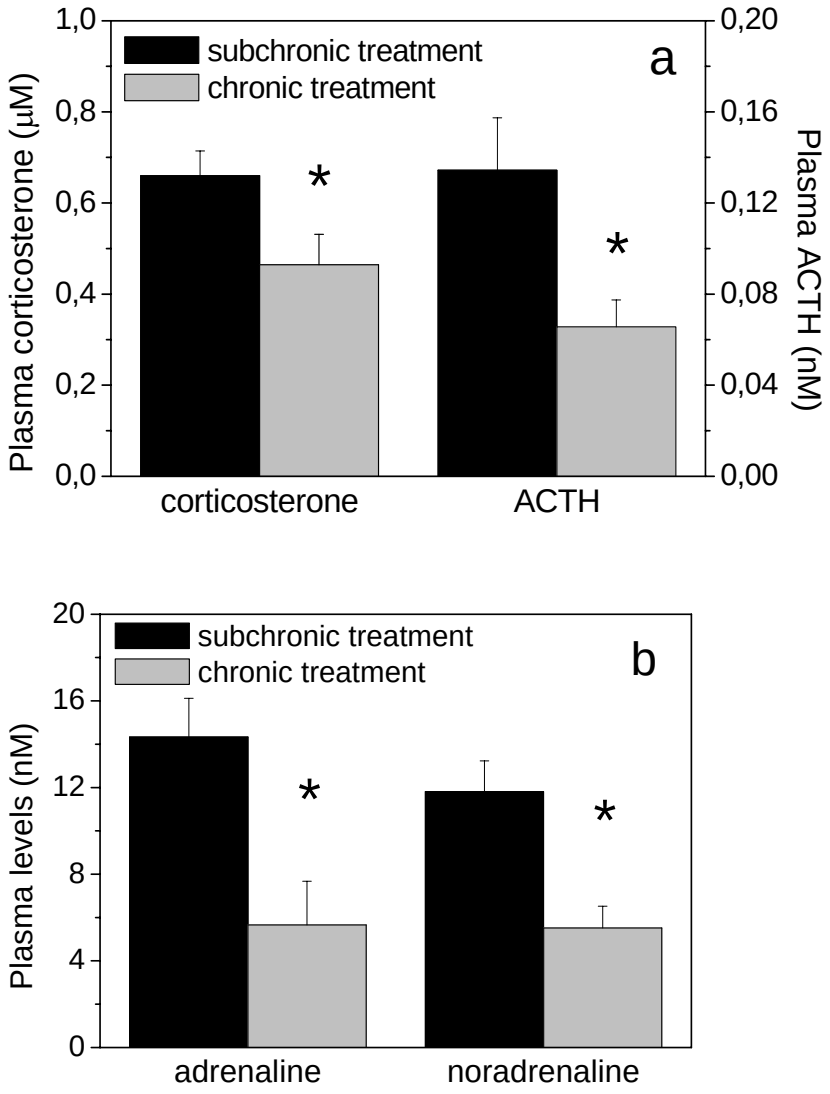

***Fig. 4a and b**. Effect of treatment on plasma levels of corticosterone and ACTH (a) and noradrenaline and adrenaline (b). Subchronic (24h) treated animals black bars (n = 9); Chronic treated animals light gray bars (n = 6). * P < 0.05, ** P < 0.01. All experiments were carried out in presence of subcutaneous implanted minipumps filled with citalopram.*

4. Discussion

5-HT$_{1B}$ receptor sensitivity

Early electrophysiological data suggest that both 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptors desensitize following chronic treatment with antidepressants (Blier et al., 1987; Chaput et al., 1986). Microdialysis studies were able to reproduce these findings for the 5-HT$_{1A}$ autoreceptor, but not for the 5-HT$_{1B}$ autoreceptor (Auerbach and Hjorth, 1995; Bosker et al., 1995; Cremers et al., 2000b).

This may partly be explained in terms of experimental conditions, however differences in pharmacokinetics should also be taken into account. In a previous study, it was emphasized that the plasma levels of citalopram during chronic treatment should be in the same range as those measured in patients (Cremers et al., 2000b). Another relevant factor could be the length of the washout period following chronic treatment. To exclude any possibility of interference, previous microdialysis experiments were performed in absence of the SSRI. However, receptor responsivity is a dynamic process and hence theoretically, resensitization could occur during washout. This is supported by an in-situ hybridization study showing that after 24 hours upon drug discontinuation 5-HT$_{1B}$ receptor mRNA was rapidly recovered (Anthony et al., 2000; Neumaier et al., 1996). To circumvent this problem, the present experiments were performed in the presence of citalopram, mimicking the clinical situation. In a previous study, we measured plasma levels throughout a similar treatment using minipumps delivering 0.25 mg/h citalopram, resulting in plasma levels of 0.3 μM citalopram (Cremers et al., 2000b), which is within the clinically effective range in humans (0.12-0.84 μM, (Baumann, 1992)). In the present study, brain citalopram concentrations were determined in both brain areas. Concentrations of citalopram in the brain were within the same range for chronically and sub-chronically treated animals, which enables direct comparison between the two conditions.

In presence of citalopram, augmentation of the 5-HT release with 5-HT$_{1B}$ receptor antagonist SB 224289 or mixed 5-HT$_{1B/1D}$ receptor antagonist GR 127935 was comparable in both chronic and subchronic treatment group, suggesting that the sensitivity of 5-HT$_{1B}$ receptors had not changed with duration of treatment.

These results do not differ from microdialysis studies performed in absence of the SSRI following a washout period (Auerbach and Hjorth, 1995; Bosker et al., 1995; Cremers et al., 2000b), indicating that 5-HT$_{1B}$ receptor resensitization does not occur. This is in contrast with the findings of in situ hybridization studies (Anthony et al., 2000; Neumaier et al., 1996). It must be realized, however, that mRNA and protein levels do not always correlate. Moreover, plasticity of

pre- and postsynaptic 5-HT$_{1B}$ receptors may be different, which is hard or impossible to discriminate at the level of mRNA.

Although in the present study no alteration was found of 5-HT$_{1B}$ autoreceptor responsivity, chronic treatment with citalopram significantly increased basal 5-HT levels in the dorsal hippocampus. This could be related to a diminished functionality of the 5-HT$_{1A}$ receptor mediated inhibitory control of the local 5-HT release, as demonstrated previously (Bosker et al., 2001; Cremers et al., 2000b). So despite the lack of effect on the 5-HT$_{1B}$ receptor, long-term treatment does induce several other pharmacological alterations which might be involved in the therapeutic effect of SSRIs.

HPA-axis activity

Besides changes within the central serotonergic system, chronic treatment with citalopram also changed several peripheral markers. The chronically treated animals had significantly lower plasma levels of adrenaline and noradrenaline, ACTH and corticosterone, suggesting a decreased activity of the HPA-axis. This is in agreement with the observation that chronic treatment with antidepressants restores HPA-axis hyperactivity in depressive patients (Barden et al., 1995; Inder et al., 2001) and that it reduces basal levels of corticosteroids and ACTH in rodents (Reul et al., 1993). Cortisol release in humans can be regulated by activation of 5-HT$_{1A}$ receptors. This response is blunted following chronic antidepressant therapy (Berlin et al., 1998; Lerer et al., 1999), which indicates a strong interaction between the serotonergic system and HPA axis activity and thus further supports our results.

Levels of corticosterone and catecholamines found in the subchronic treatment group are 10-fold higher than baseline levels reported in literature (Gomez et al., 1998; Reul et al., 1993), and comparable with a restraint stress induced response (Ginsberg et al., 2003). The termination procedure used in the present study induced acute stress in all animals, which likely explains the high plasma levels of stress hormones. Interestingly, stress hormone levels were significantly reduced in the chronic treatment group. Our results are in line with the observation that rats chronically treated with an SSRI have a blunted corticosterone response to citalopram (Jensen et al., 1999), as well as a reduced HPA-axis activation in response to acute stress (Reul et al., 1993). In short, the present study confirms that control of stress hormone release is affected by chronic SSRI treatment.

Conclusion

It can be concluded that as 5-HT_{1B} receptor function remains unaltered following chronic citalopram treatment, this receptor is not involved in the observed reduction of the stress hormone release. The notion that 5-HT_{1B} receptors do not desensitize in presence of citalopram suggests that the therapeutic effect of long-term antidepressant treatment could be improved by co-administration of a 5-HT_{1B} receptor antagonist. Further research should reveal how HPA-axis attenuation might play a role in achieving the therapeutic effect of SSRIs.

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CHAPTER 4

Acute and chronic effects of citalopram on postsynaptic 5-HT_{1A} receptor mediated long loop type of feedback in medial prefrontal cortex

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Submitted

Abstract

Evidence is accumulating that in addition to the autoreceptors, postsynaptic 5-HT_{1A} receptors are involved in the control of terminal 5-HT release. Previously we have demonstrated that chronic treatment with the selective serotonin reuptake inhibitor citalopram significantly diminished this control in the central nucleus of the amygdala. The aim of the present study was to investigate whether such adaptive changes also take place in the medial prefrontal cortex. Using dual probe microdialysis in dorsal raphe and medial prefrontal cortex, we were able to demonstrate that local infusion of the 5-HT_{1A} receptor agonist flesinoxan into the latter area significantly decreased extracellular 5-HT in both regions. Following chronic treatment, challenging the animals with citalopram showed a diminished effect on extracellular 5-HT in medial prefrontal cortex in the citalopram compared to the saline treatment group. In the citalopram treatment group this effect could be significantly augmented by local infusion of WAY 100635. Both observations indicate that chronic treatment with citalopram possibly sensitizes postsynaptic 5-HT_{1A} receptors in the medial prefrontal cortex.

Summarizing, the present study demonstrates that postsynaptic 5-HT_{1A} receptors in the medial prefrontal cortex are involved in the regulation of both local and raphe extracellular 5-HT and might sensitize upon chronic treatment.

1. Introduction

The clinical use of selective serotonin reuptake inhibitors (SSRIs) covers a wide range of psychiatric diseases, e.g. major depression, panic disorders, anxiety disorders and social phobia. Typically, the therapeutic effect of SSRIs is delayed for several weeks, indicating long-lasting adaptations at the cellular level. SSRIs enhance extracellular serotonin (5-HT) in the brain by blocking the 5-HT reuptake carrier. The excess of 5-HT activates the inhibitory somatodendritic 5-HT$_{1A}$ and terminal 5-HT$_{1B}$ autoreceptors, resulting in a decrease of firing and release, which limits the effect of reuptake inhibition on release in the projection areas. Following chronic SSRI treatment however, 5-HT$_{1A}$ autoreceptors become less sensitive, effectively reducing their effect on firing and release. It has been hypothesized that this gradual loss of responsiveness is correlated with the onset of therapeutic action (Blier et al., 1987a), for review see (Briley and Moret, 1993).

It is well accepted that presynaptic 5-HT$_{1A}$ autoreceptors, located on cellbodies in the brainstem nuclei, control the serotonergic release in the projection areas. However, evidence is accumulating that postsynaptic 5-HT$_{1A}$ receptors also play a role in the regulation of firing rate and release. Blier et al were the first to suggest that serotonergic neurons in the raphe nuclei were under control of postsynaptic 5-HT$_{1A}$ receptors, possibly through a long feedback loop from the projection areas to the raphe nuclei (Blier et al., 1987b). This idea was later confirmed by several other electrophysiology studies (Ceci et al., 1994; Celada et al., 2001) and supported by microdialysis (Casanovas et al., 1999; Romero et al., 1994) and neuroanatomical data (Aghajanian and Wang, 1977; Jankowski and Sesack, 2004; Peyron et al., 1998).

Bosker et al have shown that activation of postsynaptic 5-HT$_{1A}$ receptors in the central nucleus of the amygdala reduced the 5-HT release not only locally, but also in the cell body area, the nucleus of the caudal linear raphe (Bosker et al., 1997). Furthermore, the SSRI induced increase of 5-HT in the amygdala could be augmented 3-fold by simultaneous local infusion of a 5-HT$_{1A}$ antagonist, suggesting a tight control via local 5-HT$_{1A}$ receptors. In a follow up study it was demonstrated that these receptors were desensitized following chronic SSRI treatment (Bosker et al., 2001). In contrast, in the hippocampus antidepressant treatment markedly increased the effect of 5-HT$_{1A}$ blockade on the firing activity of CA3 pyramidal neurons, suggesting an increased sensitivity of the local 5-HT$_{1A}$ receptors (Haddjeri et al., 1998).

Apparently, the effect of antidepressant treatment may vary between brain areas. Since SSRIs are effective in both anxiety disorders and affective disorders, their therapeutic action might reflect local effects in different brain areas i.e. limbic and cortical regions. If so, investigating the role of

postsynaptic 5-HT_{1A} in regulating extracellular 5-HT in medial prefrontal cortex may contribute to our understanding of the mechanism underlying antidepressant effects.

The present study investigates the role of local postsynaptic 5-HT receptors in the regulation of 5-HT release in the medial prefrontal cortex, following acute as well as 14-day SSRI treatment. Using dual and single probe microdialyis, local infusion of both 5-HT_{1A} agonists and antagonists enabled us to properly assess cortical 5-HT_{1A} receptor functionality, in contrast with previous studies administering systemically which complicates differentiation between pre- and postsynaptic effects. In addition, receptor binding assays were used to assess the effect of chronic treatment on 5-HT_{1A} receptor binding and serotonin transporter (SERT) binding.

2. Materials and methods

2.1 Animals

Male Harlan rats (Zeist, Netherlands) weighing 285-320 g were housed eight per cage under standard conditions (22-24 ^{o}C, 12/12 light/dark cycle, food and water ad libitum). Following implantation of the minipump, rats were housed in pairs of two. After stereotaxic surgery and during the microdialysis experiments the rats were housed separately. All animal experiments were performed according to the governmental guidelines for care and use of laboratory animals and were approved by the Committee for Animal Research of the Medical Faculty of the Medical Faculty of the Groningen University.

2.2 Surgery

Acute experiments

Rats were anaesthetised with isoflurane anaesthesia (2,5%, 400ml/min N_2O, 600 ml/min O_2). A home made concentric microdialysis probe (i.d. 220 μm, o.d. 310 μm, AN 69, Hospal, Italy), made of polyacrylonitrile / sodium methyl sulphonate copolymer dialysis fiber was stereotaxically implanted in the PFC or DRN using the following coordinates: PFC; incisorbar at -3.3 mm (posterior: +3.4 mm, lateral: -0.8 mm, ventral from dura: –6.0 mm), exposed tiplenght was 4 mm. DRN; incisorbar at -3.3 mm (posterior: +1.2 mm, lateral: +1.4 mm, ventral from dura: -7.0 mm, angle 10^{o}) exposed tiplenght was 1.5 mm (Paxinos and Watson, 1982). The probe was secured in place with dental cement. Rats were allowed to recover for 1 day.

Chronic experiments

Osmotic minipumps (2ML2 Alzet, USA, 5 μl/h, 2 weeks) were either filled with saline or 50 mg/ml citalopram hydrobromide dissolved in saline under aseptic conditions. During isoflurane anaesthesia (2,5%, 400 ml/min N_2O, 600 ml/min O_2), minipumps were implanted subcutaneously on the left side of the back of the rat. After 14 days, the osmotic minipumps were removed and the remaining subcutaneous cavity was flushed twice with 5 ml of sterile saline. Hereafter, a microdialysis probe was inserted (see acute experiments). In a subset of animals the effect of chronic treatment on 5-HT$_{1A}$ and SERT binding potential was determined in the medial prefrontal cortex. The animals received a 14–day treatment with citalopram or saline, animals were terminated immediately or after a washout period of 48 hrs. Following decapitation, the medial prefrontal cortex was dissected, frozen on dry ice and stored at –80 ^{o}C.

2.3 Microdialysis experiments

Microdialysis experiments were performed 48 hrs after stereotaxic surgery. In the single probe studies, both sampling and local administration were performed in the PFC. In the dual probe studies, sampling was done in both the DRN and PFC while local administration took place only in the PFC. The probes were perfused with Ringer solution (147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, pH 6-7), using a CMA /102 microdialysis pump at a constant flow rate of 1.5 μl/min. After a stabilization period of two hours, 15 min samples were collected into vials containing 7.5 μl 0.02 M acetic acid to prevent oxidation. All experiments were performed in conscious and freely moving animals.

2.4 Drugs

The following drugs were used: Citalopram hydrobromide (kindly donated by Lundbeck (Denmark) courtesy Dr. Sanchez), and [^{3}H]MADAM (85 Ci/mmol) (kindly donated by Lundbeck (Denmark) courtesy Dr. Brennum), flesinoxan (kindly donated by Solvay Pharmaceuticals, the Netherlands), WAY 100.635 oxalate (synthesized at our medical chemistry laboratory) and [^{3}H]-8-OH-DPAT (202 Ci/mmol) (purchased from Amersham Biosciences).

2.5 Analytical procedures

2.5.1. Serotonin

Analysis of 5-HT was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 μl samples were injected into a HPLC (Shimadzu, LC-10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3 μm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector (ANTEC Leyden, Leiden, the Netherlands) at a potential setting of 500 mV vs. Ag/AgCl reference electrode. Chromatography was performed at 30^o C using the integrated column oven of the ANTEC potentiostate.

The mobile phase consisted of 4.1 g/l Na acetate , 50 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 4.5% methanol, 30 μl triethylamine, adjusted to pH 4.65 with diluted acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/sample (signal to noise ratio 2)

2.5.2. [^{3}H]-8-OH-DPAT and [^{3}H]-MADAM binding

Rat cortical tissue (36 mg/ml) was homogenized in ice-cold 50 mM Tris-HCl buffer pH 7.4 and centrifuged at 13,000 rpm and 4 ^{o}C for 15 min. The pellet was washed twice and resuspended in ice-cold Tris-HCl buffer. Endogenous 5-HT was removed by incubating the receptor suspension for 10 min at 37 ^{o}C. After centrifugation and subsequent washings (twice), the pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4, quickly frozen using liquid nitrogen and stored at –80 °C before use in radioligand binding experiments.

2.5.2.1 [^{3}H]-8-OH-DPAT

Receptor binding for the 5-HT$_{1A}$ receptor was measured using a final concentration of 0.7 nM [^{3}H]-8-OH-DPAT in presence and absence of 10 µM (+)-8-OH-DPAT. The receptor suspension was used undiluted. The assay buffer consisted of 50 mM Tris-HCl pH 7.4 supplemented with 1 mM $MnCl_2$, as described previously (Hall et al., 1985). After incubation for 20 min at 37 ^{o}C, the assay was stopped using filtration over GF-B filters (multiscreen FB opaque plates, Millipore, Bedford, MA, USA). The filters were rinsed twice, punched out of the plate, transferred into 6 ml scintillation counting vials and dispersed in 3.5 ml scintillation liquid (Ultima Gold, Packard Biosciences, Groningen, The Netherlands). The vials were shaken for 2h and counted for 5 min in a Tri-Carb 4450 liquid scintillation counter (Packard, Downers Grove, IL, USA).

2.5.2.2 [^{3}H]-MADAM

The serotonin transporter binding was determined in receptor suspensions, diluted to 100 µg protein, by incubation with a final concentration of 0.4 nM [^{3}H]-MADAM in presence and absence of 1 µM MADAM for 90 min at 22 °C. The assay buffer consisted of final concentrations of 120 mM NaCl and 5 mM KCl in 50 mM Tris-HCl buffer pH 7.4 (Chalon et al., 2003). The incubation was ended by filtration over GF-B filter prewetted with 0.05% PEI. The filters were treated as described for 5-HT$_{1A}$ binding (2.5.2.1.).

2.6 Histology

Under pentobarbital anaesthesia, the animals were killed by decapitation and the brains removed and fixed in a 5% formaldehyde solution. Correct placement of the implanted canullas was verified by histology of the brains.

2.7 Statistics

The data are presented as percentages of basal values calculated as individual means of the first four consecutive microdialysis samples. Basal values are presented as avarege ± SEM. Statistical analysis was performed using Sigmastat for windows (Jandel Software, SPSS Inc., Chicago, IL,

USA). Treatment effects were evaluated using one way ANOVA for repeated measurements, followed by Dunnet's test or two way ANOVA for repeated measurements, followed by Student-Newman-Keuls test. Level of significance was set at $p < 0.05$.

3. Results

3.1 Basal conditions

Basal levels of extracellular 5-HT in the PFC of untreated animals were 3.84 ± 0.55 (n = 40) fmol/sample. In the DRN, basal levels were 12.36 ± 3.24 (n = 10) fmol/sample. Following chronic citalopram treatment, basal levels in the PFC (5.87 ± 1.15, n = 18) did not significantly differ from vehicle treated animals (5.37 ± 1.28 fmol/sample, n = 20) or untreated animals.

3.2 Acute experiments

Effect of flesinoxan administration in the mPFC

Dual probe experiments

Activation of the cortical feedback loop by infusion of the 5-HT$_{1A}$ agonist flesinoxan at a dose of 3 μM into the mPFC significantly decreased extracellular 5-HT both in the mPFC to about 50% (χ^2_{13} = 47.6; $p < 0.0001$) and in the dorsal raphe nucleus to 70% of basal value (χ^2_{13} = 30.5; p = 0.0043). At the highest dose of 100 μM, local flesinoxan administration into the mPFC also significantly reduced the 5-HT concentration in both dorsal raphe to 60% (χ^2_{13} = 21.4; p = 0.0444) and mPFC to 40% of basal levels (χ^2_{12} = 45.6; $p < 0.0001$) (Fig 1.).

Single probe experiments

Infusion of flesinoxan decreased local 5-HT release maximally to 45% of basal value at a dose of 3 μM (χ^2_{10} = 30.5; p = 0.0007). The highest dose of 10 μM induced a decrease to 55% (χ^2_{10} = 23.0; p = 0.0109), the lowest dose of 1 μM lowered basal levels to 75% (χ^2_{10} = 14.4; p = 0.1561, n.s.). This latter effect was significantly different from the effect of the 3 μM dose ($F_{1,109}$ = 5.60; p = 0.0455) (Fig 2.).

Effect of systemic citalopram administration

Acute subcutaneous injection of citalopram induced a significant increase of 450% of basal levels (χ^2_{10} = 77.8; $p < 0.0001$). Simultaneous infusion of 1 μM WAY into the mPFC did not further augment the increase induced by citalopram (χ^2_{10} = 42.1; $p < 0.0001$) (Fig 3.).

3.3 Chronic experiments

Effect of flesinoxan administration in the mPFC

Infusion of 3 μM of flesinoxan into the mPFC induced a decrease to 45% of basal value, this effect was unaltered by treatment but significant in all groups; citalopram treatment (χ^2_{14} = 54.1;

$p < 0.0001$), saline treatment ($\chi^2_{14} = 35.1$; $p = 0.0014$) and acute administration ($\chi^2_{10} = 30.5$; $p = 0.0007$) (Fig. 4).

Effect of systemic citalopram administration

Subcutaneous citalopram administration following chronic saline treatment induced a significant increase of extracellular 5-HT to about 430% ($\chi^2_{10} = 30.5$; $p < 0.0001$), which was comparable to the increase in 5-HT levels when citalopram was coadministrated with 1 µM WAY into the mPFC ($\chi^2_{10} = 52.9$; $p < 0.0001$). No augmentation was observed as the effect of both administrations did not differ ($F_{1, 194} = 0.17$; $p = 0.9997$) (Fig. 5).

Following chronic citalopram treatment, citalopram enhanced 5-HT levels to 280% ($\chi^2_{10} = 55.0$; $p < 0.0001$), this effect was significantly decreased compared to the citalopram response in saline treated animals ($F_{1, 209} = 2.69$; $p = 0.0014$).

Simultaneous infusion of 1 µM WAY into the mPFC augmented the citalopram induced response in the citalopram treated animals to about 400% ($\chi^2_{10} = 52.9$; $p < 0.0001$), which was significantly different from the increase induced by citalopram itself ($F_{1, 224} = 2.42$; $p = 0.0040$) (Fig.6).

Effect of citalopram treatment on specific binding to the serotonin transporter and the 5-HT $_{1A}$ receptor

Chronic citalopram treatment did not have any influence on the specific binding of the citalopram analogue [^{3}H]-MADAM to cortical serotonin reuptake sides ($F_{1,17} = 0.327$; $p = 0.2611$) as demonstrated in Figure 7B. These results were unaltered by a washout period of 48 hr to ensure absence of citalopram ($F_{1,11} = 0.164$; $p = 0.6943$). The specific binding of the radiolabeled agonist [^{3}H]-8-OH-DPAT tended to increase compared to saline treated animals ($F_{1,12} = 2.55$; $p = 0.1387$) (see Figure 7A). Duration of washout period did not affect the outcome ($F_{1,8} = 0.0013$; $p = 0.9741$).

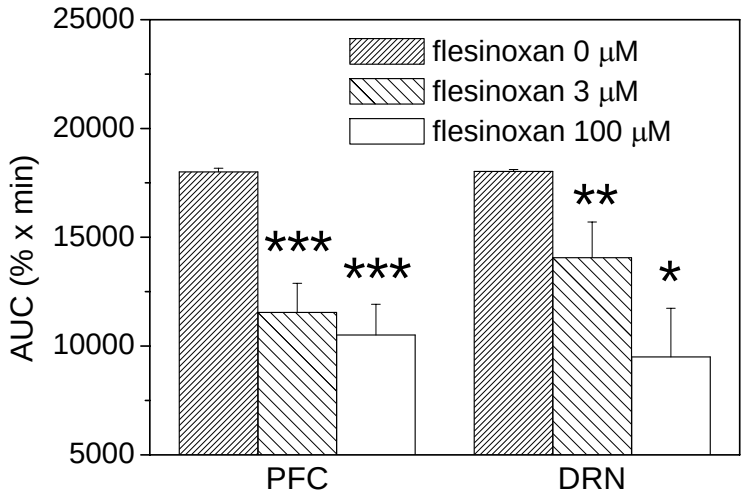

Fig. 1. *AUCs of local administration of flesinoxan at a dose of 3 and 100 μM into the mPFC on 5-HT release in the mPFC and DRN. * P<0.05, ** P<0.005, ***P<0.0001.*

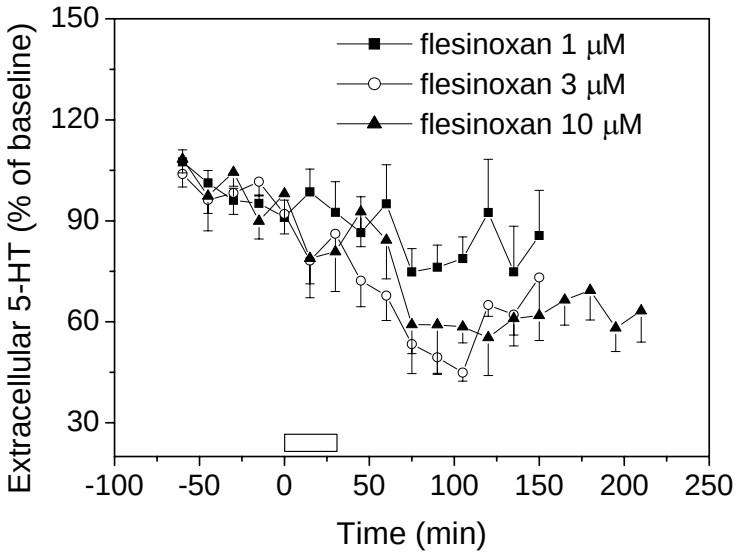

Fig. 2. *Effect of infusion of flesinoxan at a dose of 1, 3 and 10 μM into the mPFC on 5-HT release in the mPFC. filled squares = 1 μM; open circles = 3 μM; filled triangles = 10 μM.*

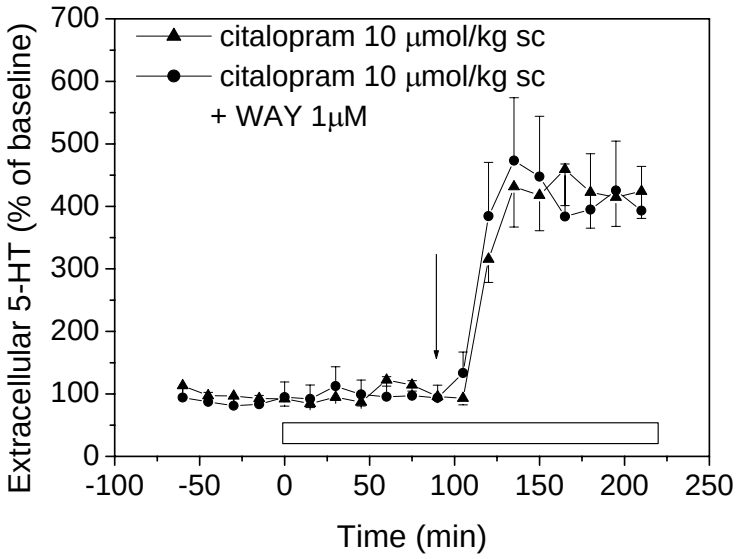

Fig. 3. *Effect of local infusion of WAY 100.635 into the mPFC on the citalopram induced response in the mPFC. Arrow depicts time point of systemic citalopram injection, open bar depicts time course of local infusion of WAY. Filled triangles = citalopram 10 μmol/kg sc; filled circels = citalopram 10 μmol/kg sc and WAY 100.635.*

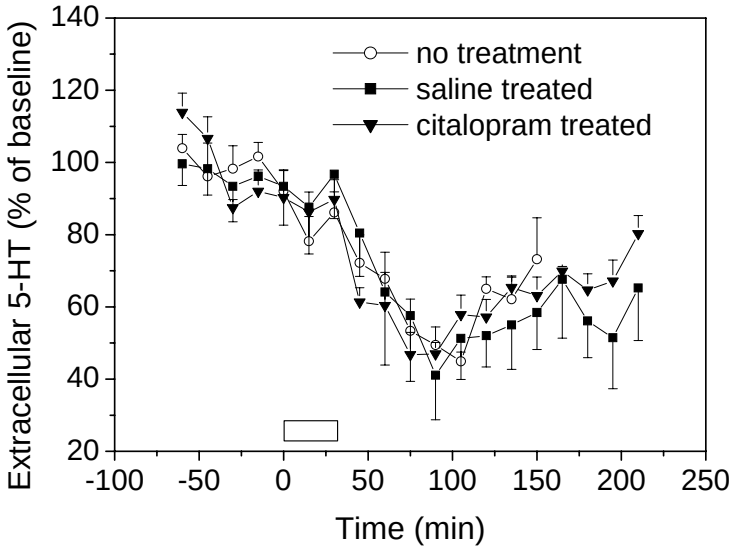

Fig. 4. *Effect of chronic citalopram treatment on the response of 5-HT release to local infusion of 3 μM flesinoxan into the mPFC. Open circles = acute; filled squares = saline treatment; filled triangles = citalopram treatment.*

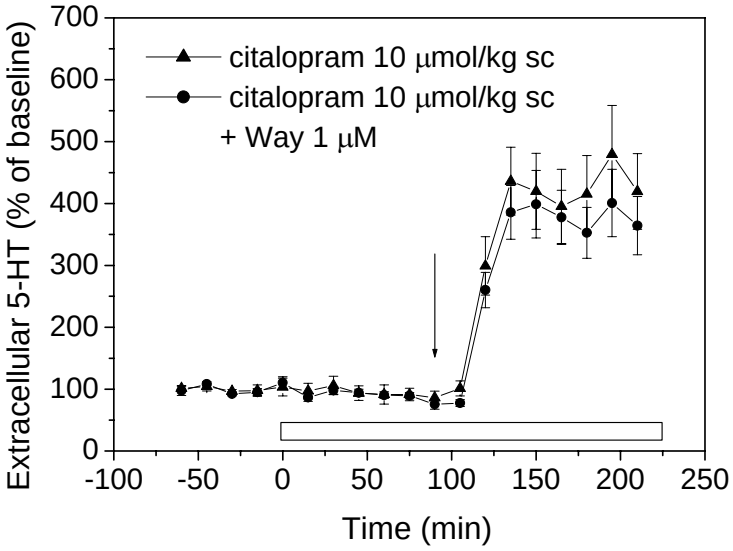

Fig. 5. *Effect of chronic saline treatment on systemic administration of citalopram and augmention by local infusion of WAY 100.635 into the mPFC. Arrow depicts time point of systemic citalopram injection, open bar depicts time course of local infusion of WAY. Filled triangles = citalopram 10 μmol/kg sc; filled circles = citalopram 10 μmol/kg sc and WAY 100.635.*

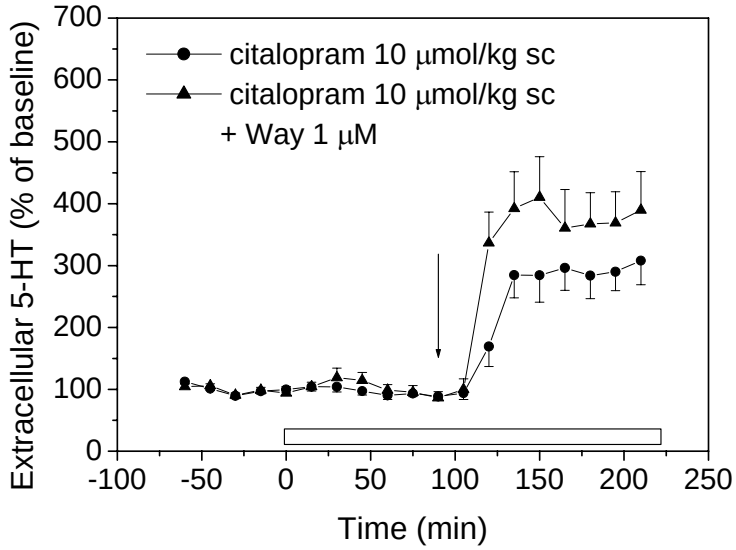

Fig. 6. *Effect of chronic citalopram treatment on systemic administration of citalopram and augmentation by local infusion of WAY 100.635 into the mPFC. Arrow depicts time point of systemic citalopram injection, open bar depicts time course of local infusion of WAY. Filled triangles = citalopram 10 μmol/kg sc; filled circles = citalopram 10 μmol/kg sc and WAY 100.635.*

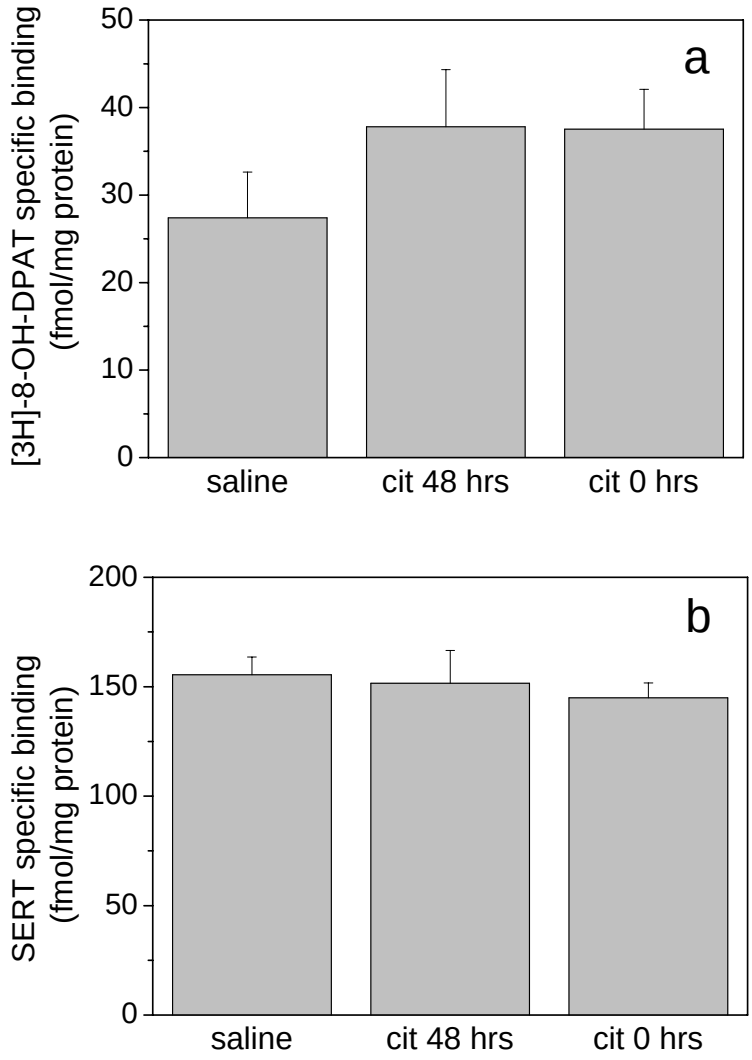

Fig. 7a and b. *Effect of chronic citalopram treatment on 5-HT $_{1A}$ receptor binding (a) and SERT binding (b). Saline = 14 day saline treatment; cit 48 hrs = 14 day citalopram treatment with 48 washout; cit 0 hrs = 14 day citalopram treatment with no washout. The specific binding was measured in triplicate.*

4. Discussion

This study provides direct evidence of postsynaptic 5-HT$_{1A}$ receptor mediated long loop type of feedback between medial prefrontal cortex and dorsal raphe nucleus. The present results also suggest that chronic treatment with citalopram increases the sensitivity of these postsynaptic 5-HT$_{1A}$ receptors.

Cortical feedback

Local infusion of flesinoxan substantially decreased cortical 5-HT release, indicating that 5-HT release in the mPFC is also controlled by local postsynaptic 5-HT$_{1A}$ receptors. The simultaneous decrease of extracellular 5-HT in the dorsal raphe suggests the presence of long loop type of feedback between the prefrontal cortex and the dorsal raphe nucleus. Such long loop type of feedback is supported by a number of neuroanatomical and electrophysiological studies (Aghajanian and Wang, 1977; Celada et al., 2001; Jankowski and Sesack, 2004; Peyron et al., 1998; Varga et al., 2001; Varga et al., 2003). Indirect evidence can also be derived from a microdialysis study in which systemically applied 8-OH-DPAT induced a larger decrease of extracellular 5-HT in the dorsal raphe compared to local administration (Tao and Auerbach, 1996). Support comes also from the observation that the decrease of extracellular 5-HT in the raphe nucleus by a systemically administered 5-HT$_{1A}$ receptor agonist could partly be prevented by simultaneous infusion of a 5-HT$_{1A}$ receptor antagonist into the mPFC (Celada et al., 2001).

Our results are in agreement with the microdialysis study reported by Celada et al. (2001) wherein local infusion of 100 μM of 8-OH-DPAT into mPFC led to a similar substantial decrease of extracellular 5-HT in both mPFC and dorsal raphe. The present study shows that this feedback loop is already activated by a relatively low concentration of 3 μM of flesinoxan.

In theory our results could be explained by diffusion of flesinoxan from mPFC to the dorsal raphe. This possibility was advanced by Jolas et al. (1995), and was based on their finding that despite lesion of hippocampal 5-HT$_{1A}$ receptors with ibotenic acid, microinjection of 8-OH-DPAT was still able to decrease 5-HT raphe firing. It is important to note that in the present study a 2000-fold lower dose was used, which makes diffusion a less likely option. Moreover, the flesinoxan induced decrease in medial prefrontal cortex could be blocked by local infusion of the 5-HT$_{1A}$ receptor antagonist WAY 100635 into the medial prefrontal cortex but not by infusion of the compound into the dorsal raphe (data not shown), which also pleads against diffusion of flesinoxan from mPFC to dorsal raphe. Hence, the present study supports previously

reported studies with respect to long loop type of feedback in mPFC (Casanovas et al., 1999; Martin-Ruiz et al., 2001) and central nucleus of the amygdala (Bosker et al., 2001).

Postsynaptic 5-HT$_{1A}$ receptor sensitivity

Acute conditions

By locally administering the 5-HT$_{1A}$ antagonist flesinoxan into the PFC, we were able to demonstrate that cortical 5-HT release is under control of postsynaptic 5-HT$_{1A}$ receptors. In a previous study we have also used an augmentation paradigm to investigate the influence of postsynaptic 5-HT$_{1A}$ receptors. Using this approach it was shown that concurrent local infusion of the 5-HT$_{1A}$ antagonist WAY in the amygdala markedly augmented the effect of systemically administered citalopram on extracellular 5-HT (Bosker et al., 2001), indicating a tight control by local 5-HT$_{1A}$ receptors. In the present study, however, local blockade of 5-HT$_{1A}$ receptors did not significantly augment the citalopram induced increase of 5-HT in the frontal cortex. In contrast, systemic co-administration of WAY strongly augmented the citalopram response in the cortex, indicating a tight control of cortical extracellular 5-HT by 5-HT$_{1A}$ autoreceptors in the raphe nuclei (Invernizzi et al., 1997).

Chronic conditions

If cortical release is predominantly controlled by raphe 5-HT$_{1A}$ receptors, one would expect that desensitization of these receptors results in increased cortical 5-HT levels. However, this appears not to be the case, since both basal 5-HT levels and the response to citalopram were not enhanced by chronic citalopram treatment. This finding is consistent with previously published chronic treatment studies in cortex (Cremers et al., 2000; Gundlah et al., 1997; Hjorth and Auerbach, 1994; Hjorth and Auerbach, 1999), suggesting that postsynaptic 5-HT$_{1A}$ receptors do not desensitize and that they might even compensate for the desensitization of raphe 5-HT$_{1A}$ autoreceptors by becoming more sensitive. The latter idea is supported by the diminished citalopram response in the chronic citalopram treatment group as compared to the chronic saline treatment group. The notion that co-infusion of 1 μM WAY was able to restore the effect of citalopram, while in the acute situation it failed to augment also points at sensitization of postsynaptic 5-HT$_{1A}$ receptors.

The failure to demonstrate this with flesinoxan is most likely related to the dose of the agonist. A concentration of 3 μM of flesinoxan already causes a near maximal response, which makes it very difficult to demonstrate a further increase in sensitivity. In contrast, augmentation with WAY 100.635 is based on the increased activation of the receptors by endogenous 5-HT as a consequence of reuptake inhibition. Arguably, such increases of 5-HT are still not able to

maximally activate the postsynaptic 5-HT$_{1A}$ receptors, which makes it easier to demonstrate a further increase in receptor sensitivity.

An increase of sensitivity was also observed when the antagonist was co-administered systemically (Gundlah et al., 1997; Hjorth and Auerbach, 1999), which is compatible with the present study. It is noteworthy that antidepressant treatment markedly increased the effect of 5-HT$_{1A}$ blockade on the firing activity of hippocampal CA3 pyramidal neurons, which also suggests an increased sensitivity of local postsynaptic 5-HT$_{1A}$ receptors (Haddjeri et al., 1998). It may seem peculiar that chronic treatment with an SSRI induces opposite effects on postsynaptic and presynaptic 5-HT$_{1A}$ receptors, but the idea is also supported by a recent study wherein chronic SSRI treatment resulted in opposite changes in capacity of the 5-HT $_{1A}$ receptor to activate its G-protein; agonist stimulated GTPγS binding was increased in the forebrain regions while decreased in the raphe nucleus (Castro et al., 2003).

Although in literature chronic SSRI treatment does not seem to affect either density (Hensler, 2002; Le Poul et al., 1995) or affinity (Castro et al., 2003; Li et al., 1997; Yocca et al., 1992) of 5-HT$_{1A}$ receptors in both raphe nuclei and forebrain, we found a trend toward increased [^{3}H]-8-OH-DPAT binding in the prefrontal cortex. If confirmed by future research, this could be a satisfactory explanation for the supersensitivity observed in the present study. Alternatively, the origin of supersensitivity should be found more downstream of the receptor and might be connected to the aforementioned changes in GTPγS binding (Castro et al., 2003).

[^{3}H]-MADAM binding to the serotonin transporter remained unaltered, indicating that both the transporter density as affinity for serotonin reuptake sites were unaffected by chronic treatment, which is consistent with several other studies using [^{3}H]-paroxetine (Cheetham et al., 1993; Kovachich et al., 1992).

It can be argued that the combination of desensitization and sensitization of presynaptic and postsynaptic 5-HT$_{1A}$ receptors is an important factor in the clinical efficacy of antidepressants, shifting control of terminal 5-HT release from the autoreceptors to their postsynaptic counterparts. Alternatively, if postsynaptic activity is not involved in the antidepressant effect, adding a 5-HT$_{1A}$ antagonist to ongoing antidepressant treatment might improve therapeutic efficacy by further enhancing extracellular 5-HT levels (see fig. 6).

Conclusion

This study provides direct evidence for the existence of a long feedback loop from the mPFC to the dorsal raphe nucleus, regulated by 5-HT_{1A} receptors in the cortex, which become increasingly sensitized upon chronic SSRI treatment. It can be argued that clinical efficacy of antidepressants is at least partly connected to an increased sensitivity of postsynaptic 5-HT_{1A} receptors induced by chronic antidepressant treatment.

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CHAPTER 5

Is tryptophan a critical factor in SSRI treatment?

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Submitted

Abstract

It is generally believed that the antidepressant effect of selective serotonin reuptake inhibitors depends on their capacity to increase extracellular serotonin. However, a limiting factor could be the availability of the serotonin precursor tryptophan. This is supported by the observation that depressed patients successfully treated with SSRIs suffer from a relapse when depleted from tryptophan. The present study has investigated the role of precursor availability as well as *de novo* synthesis in the response to the SSRI citalopram. Tryptophan depletion following oral administration of an aminoacid mixture free of the precursor or inhibition of serotonin synthesis by NSD 1015 markedly reduced the effect of citalopram on extracellular serotonin levels. Conversely, tryptophan supplementation was able to augment the effect of citalopram, even when co-administered with a 5-HT $_{1A}$, $_{1B}$ or $_{2C}$ antagonist. These data indicate a critical role of tryptophan in SSRI based treatments.

1. Introduction

In the past four decades, antidepressant research has been based mainly on the monoamine hypothesis, assuming that the biochemical origin of depression can be found in a shortage of central serotonin. This has led to the development of the selective serotonin reuptake inhibitors (SSRIs), which, although claimed to be safer, do not exceed the therapeutic efficacy of their predecessors. Problems with antidepressants involve their moderate effectiveness compared to placebo, the considerable non-response rate and the late onset of action. However, there is a large body of evidence that the antidepressant effect can be augmented by simultaneously blocking serotonergic autoreceptors. A limiting factor for both the SSRI response as well as its augmentation could be serotonin itself. The rate of synthesis of cerebral serotonin depends on the availability of its precursor tryptophan, which might limit the therapeutic efficacy of antidepressants if insufficiently present. Levels of circulating tryptophan are to a large extent determined by dietary intake and catabolism. Persistently low tryptophan levels may form a risk to develop psychopathologies, including depression, aggressive behavior and failure of impulse control. Following acute depletion of tryptophan similar symptoms may emerge. Depressed patients treated successfully with SSRIs have been reported to suffer from a short-lasting relapse, concomitant with an acute and transient depletion of tryptophan (Delgado et al., 1990) (for review see (Bell et al., 2001; Reilly et al., 1997)), emphasizing that the antidepressant response is dependent on the continuous availability of the 5-HT precursor. These observations indicate that tryptophan plays a crucial role in the therapeutic efficacy of SSRIs. Tryptophan itself also has antidepressant potential but its clinical efficacy has as yet not been established and current antidepressants are thought to be sufficiently save and effective (Shaw et al., 2002).

The delayed therapeutic response to SSRIs is generally linked to a gradual desensitization of inhibitory 5-HT autoreceptors. In absence of this control, reuptake inhibition will result in increased levels of extracellular 5-HT. Accordingly, blockade of the 5-HT autoreceptors instantaneously augments the SSRI induced increase of 5-HT (Cremers et al., 2000; Hjorth, 1993; Invernizzi et al., 1997; Rollema et al., 1996), and may thus accelerate the clinical response.

Rodent studies indicate that, in addition to autoreceptor control, 5-HT release strongly depends on precursor availability (Schaechter and Wurtman, 1989; Westerink and Devries, 1991). Trytophan depletion by either a tryptophan free diet or administration of a trytophan free amino acid drink resulted in decreased central 5-HT levels in rodents (Fadda et al., 2000; Lieben et al., 2004), which is in line with trytophan depletion studies in patients. Conversely, enhanced levels of tryptophan increase basal release and the SSRI induced 5-HT response (Gartside et al., 1992;

Perry and Fuller, 1993), emphasizing the need for exploring the use of tryptophan in antidepressant therapy.

The current study was undertaken to assess the dependency of antidepressant induced intra-cerebral release of 5-HT on the synthesis of the amine from circulating tryptophan. Accordingly, the release of 5-HT in the presence of the SSRI citalopram was measured under conditions of low and high levels of tryptophan and following local inhibition of 5-HT-synthesis. In addition, the consequences of tryptophan on augmentation of the SSRI citalopram were investigated using 5-HT $_{1A, 1B}$ and $_{2C}$ antagonists. Extracellular 5-HT as measured by microdialysis in the ventral hippocampus of the freely moving rat was used as output parameter. The clinical potential of our approach and results are discussed.

2. Materials and methods

2.1 Animals

Male Harlan rats (Zeist, Netherlands) weighing 285-320 g were housed eight per cage under standard conditions (22-24 ^{o}C, 12/12 light/dark cycle, food and water ad libitum). After stereotaxic surgery and during the microdialysis experiments the rats were housed separately. All animal experiments were performed according to the governmental guidelines for care and use of laboratory animals and were approved by the Committee for Animal Research of the Medical Faculty of the Groningen University.

2.2 Surgery

Rats were anaesthetized with isoflurane anaesthesia (2,5%, 400ml/min N_2O, 600 ml/min O_2).

Stereotaxic surgery

A home made concentric microdialysis probe (i.d. 220 μm, o.d. 310 μm, AN 69, Hospal, Italy), made of polyacrylonitrile / sodium methyl sulphonate copolymer dialysis fiber was stereotaxically implanted in the ventral hippocampus (vHC) using the following coordinates: incisorbar at -3.3 mm (posterior: -5.3 mm, lateral: +4.8 mm, ventral from dura: –8.0 mm), exposed tip length was 4 mm. (Paxinos and Watson, 1982). The probe was secured in place with dental cement. Rats were allowed to recover for 1 day.

Vena jugularis cannulation

A home made cannula was implanted in the jugular vein. The tubing was tunneled subcutaneously to the head and attached to the scull. Animals were allowed to recover at least 24 hours. If the animals were not in experiment, the canulla was filled with a saline/heparin solution containing poly vinyl pyridine.

2.3 Experiments

Microdialysis

Microdialysis experiments were performed 24 hrs after stereotaxic surgery. Animals which were administered an amino acid mixture were deprived from food 12 hrs before the start of the experiment in order to minimize tryptophan uptake from food.

The probes were perfused with Ringer solution (147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, pH 6-7), using a CMA /102 microdialysis pump at a constant flow rate of 1.5 μl/min. After a stabilization period of two hours, 15 min samples were collected into vials containing 7.5 μl 0.02

M acetic acid to prevent oxidation. All experiments were performed in conscious and freely moving animals.

Blood sampling

Before the start of the experiment, the jugular vein canulla was flushed with a 15 IU heparin/ saline solution. Animals were connected to a Dilab Accusampler (Dilab, Sweden) programmed to take 8 blood samples during the experiment. Samples were immediately centrifuged at 14,000 rpm for 5 min and stored at –20 ^{o}C till analysis.

2.4 Drugs

The following drugs were used: Citalopram hydrobromide (kindly donated by Lundbeck (Denmark) courtesy Dr. Sanchez), tryptophan methylester, NSD 1015 and SB 204648 (purchased from Sigma-Aldrich), WAY 100.635 oxalate and GR 129735 (synthesized at our medical chemistry laboratory). The amino acid carbohydrate mixture consisted of Solugel P and maltodextrine. Gelatin hydrolysate (Solugel P®) was purchased from PB Gelatins (Tessenderlo, Belgium; see Table 1 for amino acid composition). Maltodextrine was obtained from the Amylumgroup (Koog aan de Zaan, The Netherlands).

2.5 Analytical procedures

2.5.1 5-HT

Analysis of 5-HT was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 µl samples were injected into a HPLC (Shimadzu, LC-10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3 µm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector (ANTEC Leyden, Leiden, the Netherlands) at a potential setting of 500 mV vs. Ag/AgCl reference electrode. Chromatography was performed at 30 ^{o}C using the integrated column oven of the ANTEC potentiostat.

The mobile phase consisted of 4.1 g/l Na acetate, 50 mg/l heptane sulphonic acid sodium salt, 500 mg/l EDTA, 4.5% methanol, 30 µl triethylamine, adjusted to pH 4.65 with diluted acetic acid. The flow rate was 0.4 ml/min. The detection limit for 5-HT was 0.5 fmol/sample (signal to noise ratio 2).

2.5.2 Analysis tryptophan

Plasma was analyzed according to Kema et al. (Kema et al., 2001).

2.6 Histology

Under pentobarbital anaesthesia, the animals were killed by decapitation and the brains removed and fixed in a 5% formaldehyde solution. Correct placement of the implanted canullas was verified by histology of the brains.

2.7 Statistics

The data are presented as percentages of basal values calculated as individuals means of the first four consecutive microdialysis samples. Statistical analysis was performed using Sigmastat for windows (Jandel Software, SPSS Inc., Chicago, IL, USA). Treatment effects were evaluated as treatment x time effects using two way ANOVA for repeated measurements, followed by Student-Newman-Keuls test. Level of significance was set at $p < 0.05$.

3. Results

Basal conditions

Basal levels of extracellular 5-HT in the ventral hippocampus were 5.40 ± 0.34 fmol/sample (N = 86). Basal levels of animals deprived from food 12 hrs before the experiment did not significantly differ from basal levels of rats feeded ad libitum (4.82 ± 0.89, N = 9).

Effect of tryptophan depletion on citalopram response

Administration of citalopram at a dose of 10 µmol/kg increased basal 5-HT levels to about 540% (Fig. 3a). Oral administration of the amino acid mixture with a regular amount of tryptophan (0.28%) did not significantly affect the citalopram induced 5-HT response. Precursor depletion by the same amino acid mixture without trytophan did not significantly affect basal levels of 5-HT but diminished the effect of citalopram to 375% ($F_{1,142} = 3.33$; $p = 0.0004$) (Fig. 1).

Effect of NSD infusion on 5-HT release and citalopram response

Inhibition of synthesis by local infusion of the amino acid decarboxylase inhibitor NSD 1015, which blocks the enzymatic conversion of 5-HTP into 5-HT, strongly reduced basal levels of 5-HT to about 40% ($F_{1, 95} = 5.42$; $p < 0.0001$). Subsequently, the effect of citalopram was also diminished more than two-fold when 5-HT synthesis was inhibited ($F\ 1,143 = 9.91$; $p < 0.0001$) (Fig. 2).

Effect of trytophan addition on citalopram response

The effect of citalopram on 5-HT was dose dependently augmented by addition of tryptophan. A dose of 30 mg/kg significantly enhanced the citalopram response from 540% to 1000% ($F_{1,272} = 8.47$; $p < 0.0001$, a higher dose of 100 mg/kg increased 5-HT levels further to 1200% ($F_{1,251} = 6.94$; $p < 0.0001$), the difference in augmentation between both doses was not significant ($F_{1,272} = 0.887$; $p = 0.6037$) (Fig. 3a).

Effect of antagonist co-administration on citalopram response

Co-administration of the 5-HT_{1A} antagonist WAY at a dose of 1 µmol/kg slightly but significantly enhanced the citalopram induced increase of 5-HT to 640% of basal levels ($F_{1,209} = 1.986$; $p = 0.0104$). Blocking the 5-HT_{2C} receptors with 1 µmol/kg SB augmented the effect of citalopram to 740%, which was not significant ($F_{1,209} = 0.947$; $p = 0.5307$). Augmentation with

the 5-$HT_{1B/\ 1D}$ antagonist GR significantly enlarged the citalopram response to 700% ($F_{1,272}$ = 2.11; p = 0.0047) (Figs. 3a, b, c and d).

Effect of antagonist co-administration on trytophan induced augmentation

Blockade of the 5-HT_{1A} receptors by 1 μmol/kg of WAY did not influence augmentation of the citalopram response by tryptophan at both doses of 30 mg/kg ($F_{1,272}$ = 0.487; p = 0.9696) and 100 mg/kg ($F_{1,251}$ = 0.266; p = 0.9994) (Fig. 3b).

Although blocking the 5-HT_{2C} receptors by co-administration of SB modestly increased the augmentation effect of tryptophan to 1200% and 1300% at a dose of 30 mg/kg and 100 mg/kg respectively, these effects were not significant ($F_{1,293}$ = 0.443; p = 0.9827; $F_{1,272}$ = 0.2402; p = 0.9997, respectively) (Fig. 3c).

The 5-$HT_{1B/\ 1D}$ antagonist GR at a dose of 1 μmol/kg did significantly increase the tryptophan mediated augmentation of 30 mg/kg to 1300% ($F_{1,272}$ = 2.16; p = 0.0037) and 100 mg/kg to 1900% ($F_{1,251}$ = 2.93; $p < 0.001$) (Fig. 3d).

Effect of tryptophan on plasma levels

Basal levels of total plasma tryptophan were about 50 μmol/L. Administration of tryptophan at a dose of 30 mg/kg resulted in a significant increase of plasma levels to 125 μmol/L (P = 0.0179), a higher dose of 100 mg/kg induced a maximum of 240 μmol/L (P = 0.0286) (Fig. 4).

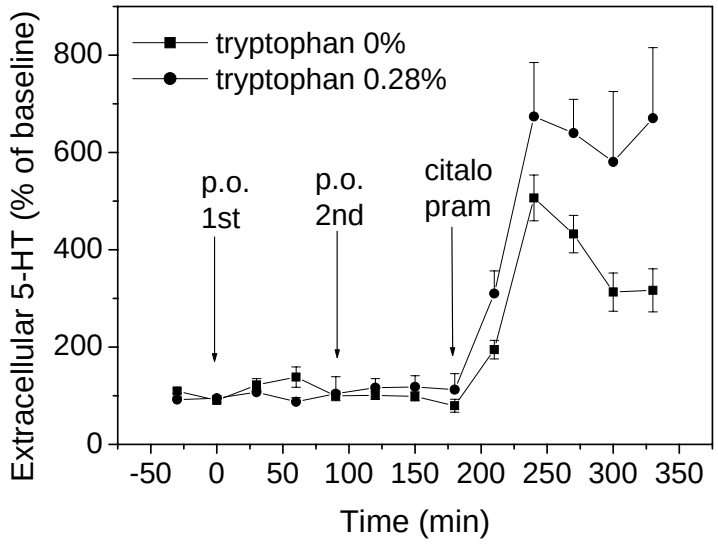

Fig. 1. *The effect of oral tryptophan depletion on the response to citalopram. Filled squares; conditions of low tryptophan, filled circles; conditions of normal tryptophan. First arrow at t=0; first oral administration of aminoacid mixture (for content, see table 1.); second arrow at t=90; second oral administration; third arrow at t=180; subcutaneous administration of citalopram 10 µmol/kg.*

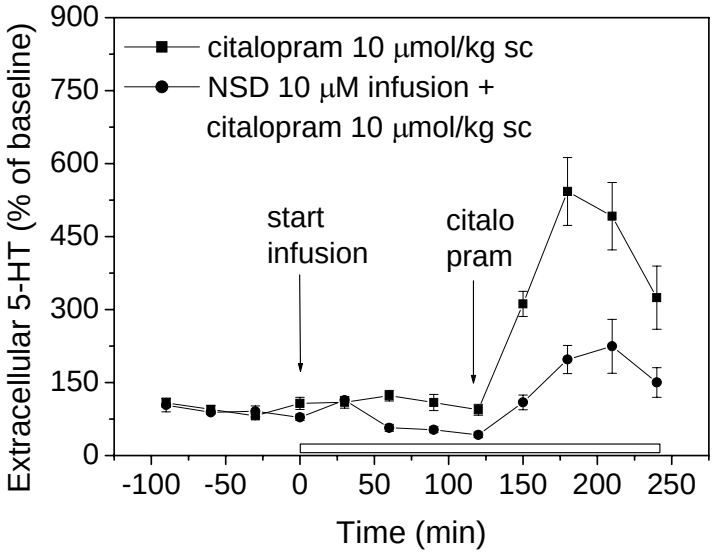

Fig. 2*. The effect of blockade of the 5-HT synthesis on the response to citalopram. Filled squares; no synthesis inhibition, t=120 citalopram 10 µmol/kg sc, filled circles; synthesis inhibition by infusion of NSD 10 µM at t=0, t=120 citalopram 10 µmol/kg sc.*

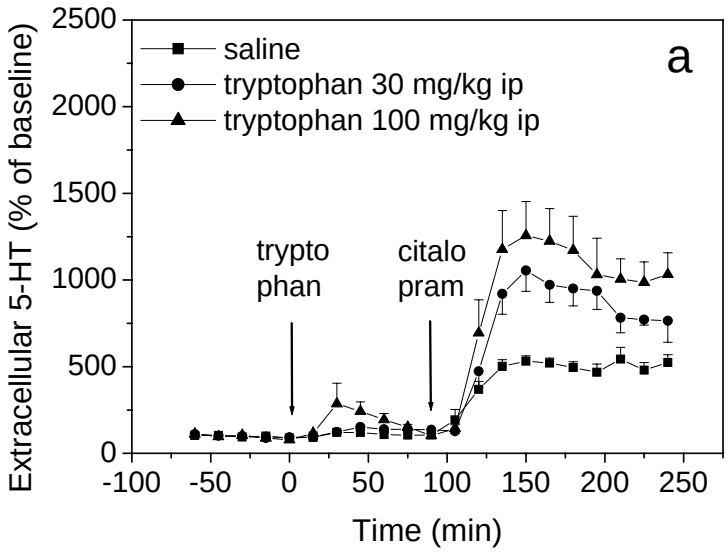

Fig. 3a. *The effect of tryptophan on the response to citalopram. Filled squares; t=90 citalopram 10 µmol/kg sc, filled circles; t=0 trytophan 30 mg/kg ip, t=90 citalopram 10 µmol/kg sc, filled triangles; t=0 trytophan 100 mg/kg ip, t=90 citalopram 10 µmol/kg sc.*

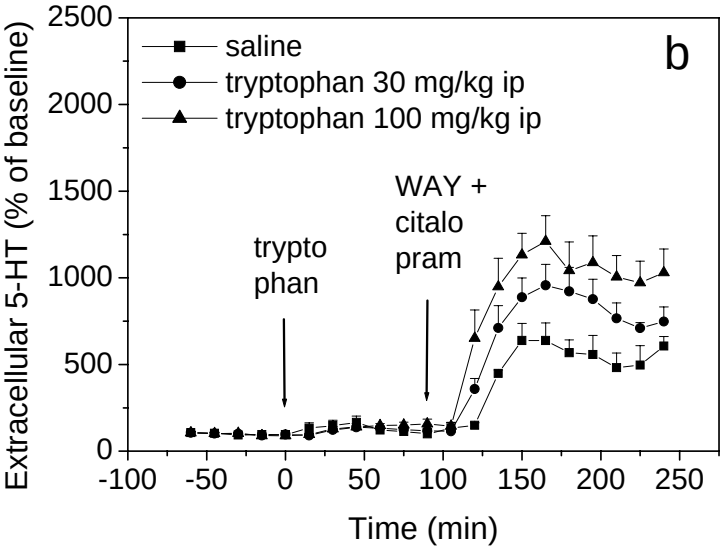

Fig. 3b. *The effect of WAY 100635 1 µmol/kg sc on tryptophan induced augmentation of the response to citalopram. t=90 citalopram 10 µmol/kg sc and WAY 1 µmol/kg sc. Filled squares; t=0 saline ip, filled circles; t=0 trytophan 30 mg/kg ip, filled triangles; t=0 trytophan 100 mg/kg ip.*

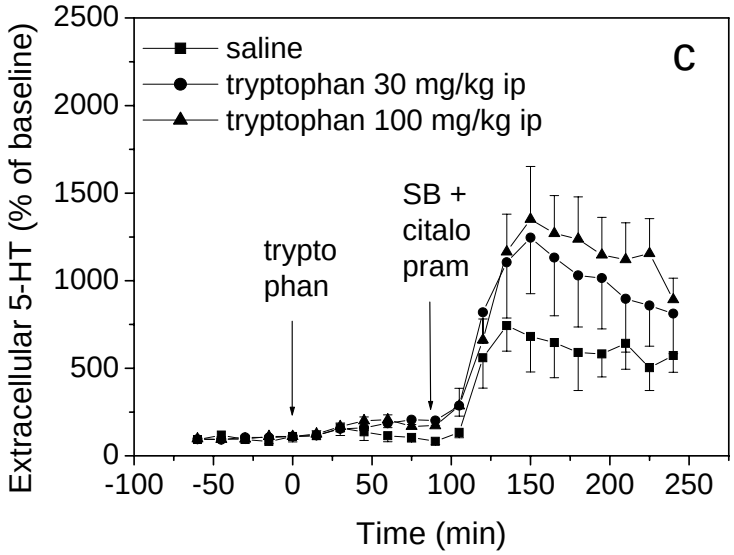

Fig. 3c. *The effect of SB 204648 1 µmol/kg sc on tryptophan induced augmentation of the response to citalopram. t=90 citalopram 10 µmol/kg sc and SB 204648 1 µmol/kg sc. Filled squares; t=0 saline ip, filled circles; t=0 trytophan 30 mg/kg ip, filled triangles; t=0 trytophan 100 mg/kg ip.*

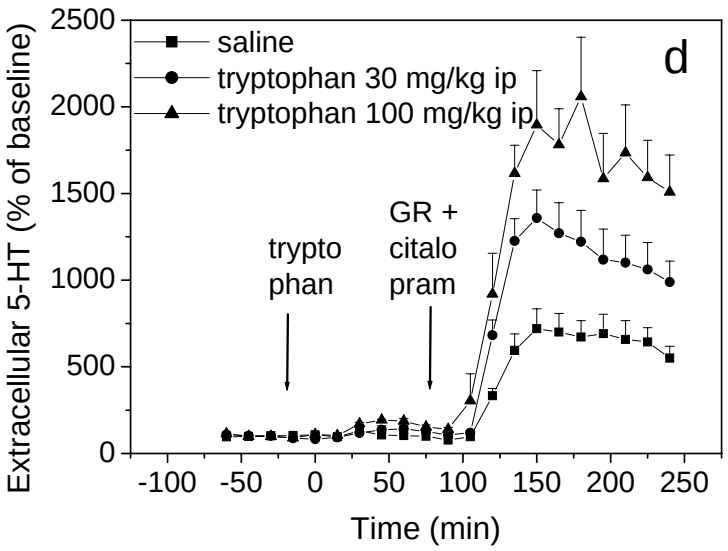

Fig. 3d. *The effect of GR 129735 1 µmol/kg sc on tryptophan induced augmentation of the response to citalopram. t=90 citalopram 10 µmol/kg sc and GR 129735 1 µmol/kg sc. Filled squares; t=0 saline ip, filled circles; t=0 trytophan 30 mg/kg ip, filled triangles; t=0 trytophan 100 mg/kg ip.*

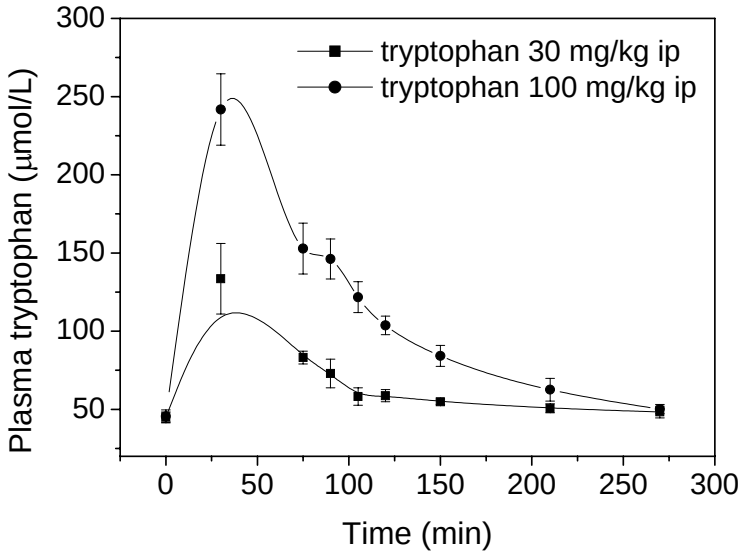

Fig. 4. *Tryptophan plasma curve following intraperitoneal administration. Filled squares; tryptophan 30 mg/kg ip, filled circles; tryptophan 100 mg ip.*

Protein (Solugel P®) in 100 ml ultra pure water	**100**
Aspartic acid + asparagine	5.2
Glutamic acid + glutamine	9.3
Hydroxyproline	12.1
Serine	3.1
Glycine	22.5
Histidine	0.5
Arginine	8.8
Threonine	1.1
Alanine	9.3
Proline	13.3
Tyrosine	0.4
Valine	2.1
Methionine	0.6
Cysteine	0.2
Isoleucine	1.4
Leucine	3
Hydroxylysine	1.4
Phenylalanine	1.9
Tryptophan	0.1
Lysine	3.6
Carbohydrate (Malthodextrine) in 80 ml ultra pure water	**50**
KCl	**0.094**
CaCl2·2H2O	**2.32**
l-Tryptophan (Tryp- group)	**0**
l-Tryptophan (Tryp+ group)	**0.28**

Table 1. *Composition of the nutritional mixture and determination of the amino acids content of the gelatin-based protein (g). The composition of the nutritional mixture used in this experiment is described in bold. The amino acid spectrum (%) of the Solugel P® protein.*

4. Discussion

The present study supports the clinical observation that the therapeutic efficacy of antidepressants highly depends on the availability of tryptophan (Delgado et al., 1990; Miller et al., 1992). Our results show that depletion of tryptophan halved the effect of the SSRI citalopram (Fig. 1). This is in line with other animal studies demonstrating a substantial decline of 5-HT release (Fadda et al., 2000; Stancampiano et al., 1997) and a diminished response to serotonergic drugs like SSRIs under conditions of low levels of tryptophan (van der Stelt et al., 2004).

Similarly, inhibition of serotonin synthesis by NSD 1015 strongly reduces the effect of citalopram, which indicates that the effect of SSRIs largely depends on *de novo* synthesis of serotonin. It is therefore conceivable that insufficient synthesis of serotonin, for instance caused by an unfavorable tryptophan hydroxylase TPH-2 gene polymorphism (Zhang et al., 2004; Zhang et al., 2005; Zill et al., 2004), can contribute to the high non-response rates with SSRI treatment.

Recently, in a meta-analysis of 108 clinical trials using tryptophan or 5-HTP, it was concluded that both serotonin precursors could be used to treat depression (Shaw et al., 2002). Their use in clinical practice is however restricted by the fear of severe side effects like the serotonergic syndrome (Steiner and Fontaine, 1986). Nevertheless, preclinical evidence does demonstrate a direct enhancing effect of enlarged plasma tryptophan on central serotonergic release and synthesis (Gartside et al., 1992; Perry and Fuller, 1993; Westerink and Devries, 1991), which can offer an alternative way to ameliorate the effectiveness of current antidepressants.

The present results show an augmented response to the SSRI citalopram when co-administered with tryptophan (Fig. 3a), which is in agreement with previous reports (Dreshfield-Ahmad et al., 2000; Gartside et al., 1992; Perry and Fuller, 1993). However, while the use of tryptophan in antidepressant treatment is described in both clinical literature and animal studies, little attention has been paid to its kinetics. Severe side effects like the serotonergic syndrome using tryptophan or 5-HTP in the clinic might have occurred due to relative high dosages used, as excessive peripheral levels of tryptophan do influence central levels of serotonin (Mitchell, 1997; Sporer, 1995). However, the need for such high levels of tryptophan seems questionable. The present results show that a rather small increase of tryptophan plasma levels markedly increases the effect of citalopram (Fig. 3a), suggesting that relatively low doses of tryptophan might already be sufficient.

Basal plasma levels of tryptophan were about 50 µmol/L, which is comparable to human tryptophan levels (Delgado et al., 1990). Increasing basal levels to a steady-state level of 60 µmol/L did augment the effect of citalopram two-fold (Fig. 4 & 3a). Although further enhancing the dose caused an additional increase in plasma levels, it did not further augment the citalopram

response. As known from literature, the rate limiting enzyme tryptophan hydroxylase, converting tryptophan into 5-hydroxy tryptophan (5-HTP), is unsaturated under normal conditions. If tryptophan levels rise, the enzyme becomes saturated and the synthesis of serotonin will reach its maximal level (Carlsson and Lindqvist, 1978). This can explain the fact that we did not observe an additional effect after increasing the dose of tryptophan. Clinically this implies that, when co-administering tryptophan, the dosage should be adjusted to an amount which increases tryptophan just below the level of enzyme saturation. In this way, side effects may be circumvented while still clinically effective. Given the fact that the therapeutic effect of antidepressants strongly depends on tryptophan availability and that low tryptophan levels are often associated with a variety of psychopathological disorders, treatment with serotonergic antidepressants should probably be adjusted to the patient's peripheral indol metabolism in order to prove successful.

Augmentation with a 5-HT receptor blocker can be used if treatment with an antidepressant appears insufficient. This concept has been clinically applied by co-administration of an SSRI with the mixed β-adrenergic and 5-HT_{1A} receptor antagonist pindolol (Ballesteros and Callado, 2004). Arguably, this may have little effect if the reason for the absent therapeutic effect originates from insufficient levels of tryptophan.

5-HT_{1A} receptor-mediated augmention depends on the dose of co-administered tryptophan, which is not different from the situation in absence of the antagonist (Figs. 3a and b). So 5-HT_{1A} receptor-mediated augmention, like the citalopram response itself, does indeed depend on tryptophan availability, but 5-HT_{1A} autoreceptors do not play a role in the tryptophan induced augmentation of citalopram. Clinically this implies that, at the dosages used in the present study, co-administration with a precursor is a more effective augmention strategy than 5-HT_{1A} receptor inhibition. This also holds for the 5-HT_{2C} receptor, as likewise, its antagonist SB did not affect the tryptophan mediated augmentation (Fig. 3c). Nevertheless, antagonism of the 5-HT_{2C} receptors has been reported to reverse the serotonin syndrome (Graudins et al., 1998; Hoes and Zeijpveld, 1996; Klaassen et al., 1998), which could be of clinical use in a tryptophan based augmentation strategy.

Inhibition of the 5-HT_{1B} receptor on the contrary did enhance the tryptophan induced augmentation of citalopram almost by a factor two (Fig. 3d). Whereas 5-HT_{1A} receptors control the firing rate of serotonergic neurons, 5-HT_{1B} receptors directly inhibit both release and synthesis upon activation. Based on our findings we can conclude that these last two processes become the limiting factor when augmenting with tryptophan. 5-HT_{1B} antagonists and tryptophan have a synergistic augmenting effect and could be combined if therapeutic response to either combination with an SSRI remains insufficient.

Conclusion

The present results emphasize that low tryptophan does impair the response to antidepressants, which could contribute to the low success rate of antidepressant treatment. In order to increase therapeutic efficiency, treatment with serotonergic antidepressants should be adjusted to the patient's peripheral indol metabolism, which might require additional tryptophan to ensure therapeutic action. Although most clinical studies using tryptophan report the use of rather high dosages, our data demonstrate that the antidepressant response can be augmented at a relatively low dose, which could prevent serious side effects. Further clinical research should reveal if this indeed is a safe and a good alternative to enhance the antidepressant effect of SSRIs.

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CHAPTER 6

Effect of chronic and acute administration of citalopram on serotonin synthesis, storage and metabolism in the rat brain

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Abstract

While extracellular serotonin is commonly used to assess the effects of long-term treatment with selective serotonin reuptake inhibitors, it is still not clear how this affects serotonergic markers like synthesis, storage and metabolism. It is conceivable that in order to maintain intracellular stores of serotonin, synthesis needs to adjust to the conditions of prolonged reuptake inhibition. In the present study, we investigated the effect of chronic SSRI treatment on serotonin, its metabolite HIAA and the precursor 5-hydroxy tryptophan in tissue of rat brain. It was found that chronic treatment resulted in a dramatic depletion of the serotonin content in the brain, which most likely is the result of insufficient serotonin synthesis caused by prolonged autoreceptor activation.

In addition, we have demonstrated that a washout period rapidly reverses the effects of chronic treatment in terminal areas only, indicating a role for the 5-HT_{1B} receptor. This might parallel the clinical phenomenon of rebound depression, which occurs when suddenly discontinuing treatment with antidepressants. These findings further support the clinical practice to slowly phase out SSRI treatment.

1. Introduction

The serotonergic system and consequently also its response to serotonergic drugs like antidepressants are known to be under tight control of a number of feedback mechanisms. Increased levels of serotonin (5-HT) caused by serotonergic reuptake inhibitors (SSRIs) activate several inhibitory autoreceptors controlling serotonergic synthesis (Moret and Briley, 1997;Barton and Hutson, 1999;Hjorth et al., 1995), release (Invernizzi et al., 1997;Rollema et al., 1996;Hjorth, 1993;Cremers et al., 2000) and turnover (Stenfors and Ross, 2002;Fuller, Perry et al., 1974). Starting chronic SSRI treatment, all processes immediately lessened, but steadily normalized or even increased during treatment (Kreiss and Lucki, 1995;Le Poul et al., 1995;Esteban et al., 1999;Stenfors and Ross, 2002), suggesting a gradually reduced functionality of the serotonergic autoreceptors. It is generally believed that the increase of extracellular serotonin as a result of diminished autoreceptor control might underlie the therapeutic response to antidepressants (Blier, de Montigny et al., 1987;Briley and Moret, 1993). Whereas the effect of long term antidepressant treatment on serotonergic release, turnover and synthesis has been investigated extensively, it still remains unknown if intracellular serotonin stores are affected. Serotonergic cells partly rely on synthesis and partly on reuptake of extracellular serotonin to maintain their intracellular concentration. So theoretically, intracellular serotonin stores could get depleted if synthesis rate remains unadjusted while reuptake is continuously blocked by chronic treatment.

In the present study, we investigated the effect of chronic citalopram treatment on brain serotonin, synthesis and metabolism in tissue of several brain areas.

The conversion of tryptophan into 5-HTP is considered to be the rate-limiting step in the synthesis of serotonin, as under normal conditions, 5-HTP is immediately converted into 5-HT by the non-specific enzyme aminoacid decarboxylase. The rate of serotonin synthesis was measured as the amount of 5-HTP accumulated when blocking this final step. The ratio of 5-HIAA/5-HT was used as an index of serotonergic metabolism or turnover.

As a marker of the serotonergic system, extracellullar serotonin measured by microdialysis is generally used to assess the effect of prolonged antidepressant treatment. However, this represents only a fraction of the total serotonin content in the brain as the amount stored intracellular is a 1000 fold higher. In the present study, tissue destruction was used to investigate effects on total levels of serotonin, HIAA and 5-HTP, which includes both intra- and extracellular levels, but merely represents the intracellular situation. Compared to extracellular measurements, this might give a better view of general changes in serotonergic homeostasis throughout the whole brain.

The effect of chronic treatment is commonly assessed by a challenge following a washout period in order to prevent pharmacological interference with the treatment. However, it has been shown that adaptive processes seen after chronic treatment can revert whithin the time span of the washout period (Neumaier, Root et al., 1996). This implies a rapid adaptation of the serotonergic system and also questions the effects observed after a washout. In the present study, citalopram was delivered by osmotic minipumps to ensure stable plasma levels and analyzed to evaluate kinetics. The effect of chronic treatment on a challenge with citalopram was studied both after a washout and in presence of the minipump. The latter could give a better insight in the effects of chronic treatment on the serotonergic homeostatis because it more closely resembles the clinical situation.

2. Materials and methods

2.1 Animals

Male Harlan rats (Zeist, Netherlands) weighing 285-320 g were housed eight per cage under standard conditions (22-24 ^{o}C, 12/12 light/dark cycle, food and water ad libitum). Following implantation of the minipump, rats were housed in pairs of two. All animal experiments were performed according to the governmental guidelines for care and use of laboratory animals and were approved by the Committee for Animal Research of the Medical Faculty of the Medical Faculty of the Groningen University.

2.2 Treatment

Osmotic minipumps (2ML2 Alzet, USA, 5 μl/h, 2 weeks) were either filled with saline or 50 mg/ml citalopram hydrobromide dissolved in saline under aseptic conditions. During isoflurane anaesthesia (2,5%, 400 ml/min N_2O, 600 ml/min O_2), minipumps were implanted subcutaneously on the left side of the back of the rat.

In the treatment group including a washout period, the osmotic minipumps were removed after 14 days, the remaining subcutaneous cavity was flushed twice with 5 ml of sterile saline and animals were sacrified 48 hours after removal of their minipump. The other treatment groups include a 14 day saline treatment and a 14 day citalopram treatment, these animals were sacrified with their minipump still in place.

At the day of the termination, animals were challenged with either citalopram 10 μmol/kg sc or saline. After 45 min. NSD 1015 was injected intraperitoneally at a dose of 100 mg/kg. Another 45 min later, animals were anaesthetized with isoflurane anaesthesia (2,5 %, 400ml/min N_2O, 600 ml/min O_2), blood was taken by cardiac puncture, brains were removed, rapidly frozen at dry ice and stored at –80 ^{o}C.

2.3 Tissue dissection

Brains were sliced on a cryostat and punches were taken from nine brain areas; anterior cingulate cortex (ACAD), nucleus accumbens (NAc), caudate putamen (CP), paraventricular nucleus of the hypothalamus (PVN), dorsal hippocampus (dHC), ventral hippocampus (vHC), central amygdala (Amy). Brain samples were homogenized with 100 μL of 0.1 M perchloric acid and centrifuged at 14000 rpm for 10 min at 4 ^{o}C. The supernatant was removed and assayed for 5-HT, 5-HIAA and 5-HTP.

2.4 Drugs

The following drugs were used: Citalopram hydrobromide (kindly donated by Lundbeck (Denmark) courtesy Dr. Sanchez) and NSD 1015 (purchased from Sigma).

2.5 Analytical procedures

2.5.1. 5-HT, 5-HIAA and 5-HTP

Analysis of 5-HT and 5-HIAA was performed by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, 20 µl samples were injected into a HPLC (Shimadzu, LC-10AD liquid chromatograph) equipped with a reversed-phase column (phenomex hypersil 3 : 3 µm, 100 x 2.0 mm, C18, Bester, Amstelveen, the Netherlands) and an electrochemical detector (ESA, Chelmsford, MA, USA) at a potential setting of 600 mV vs. Ag/AgCl reference electrode. The mobile phase consisted of 4.1 g/l Na acetate , 150 mg/l octane sulphonic acid sodium salt, 10 % methanol, adjusted to pH 4.1 with acetic acid. 5-HTP was analyzed by adjusting the methanol content to 5%. The flow rate was 1.0 ml/min.

2.5.2. Citalopram

Citalopram was measured in plasma according to Oyehaug et al. (1982) with minor modifications. Dialysate samples were injected into an HPLC (1084B Liquid Chromatograph, Hewlett Packard) which was connected with a fluorescence detector (470 Scanning Fluorescence detector, Waters, England) operating at an absorption wavelength of 240 nm, an emission wavelenght of 296 nm, and a slitwidth of 12 nm. Separation was performed using a Supelcosil HPLC column (5 µm, C18, 250 x 46 mm, Supelco, the Netherlands), at ambient temperature. The mobile phase consisted of 46% v/v acetonitrile, 54% v/v potassium dihydrogen phosphate buffer (4.3 g/l) and 1 mM tetramethylammonium, at pH 3.0. The flow rate was set at 0.75 ml/min. The detection limit was 5 nM (signal to noise ratio = 2)

2.6 Data processing and statistics

Levels are depicted as percentage of the control group, the saline treated animals receiving a saline challenge. All data are depicted in table 1, results which are discussed are presented in grahps and have been statistically analyzed. Statistical analysis was performed using Sigmastat for windows (Jandel Software, SPPS Inc., Chicago, IL, USA). Treatment or challenge effects were evaluated using one way ANOVA.

3. Results

Effect treatment on intracellular serotonin stores

Compared to saline treated animals, chronic treatment with a subsequent washout period of 48 hours reduced intracellular serotonin throughout the brain, showing a statistical difference in the NAc ($P = 0.0017$), CP ($P = 0.0063$), dHC ($P = 0.00816$) and PVN ($P = 0.0095$). Following chronic treatment without washout, this effect was even stronger, now being statistically different in Acad ($P = 0.0077$), NAc ($P = 0.0179$), CP ($P = 0.0402$), dHC ($P = 0.0044$) and PVN ($P = 0.0007$) (Fig. 1).

Effect washout on treatment

After a washout period of 48 hours, both turnover and synthesis were increased following chronic treatment. In absence of a washout period, these processes were decreased. Although a clear trend in all brainareas, this difference was significant in the Nac ($P = 0.0016$), vHC ($P = 0.0093$), Amy ($P = 0.0004$) and PVN ($P = 0.0117$) for the turnover, synthesis statistically differed in Acad ($P = 0.0257$), CP ($P = 0.0344$), dHC ($P = 0.0378$), vHC ($P = 0.0303$), PVN ($P = 0.0329$) and DRN ($P = 0.0417$) (Figs. 2 and 3).

Effect acute and chronic treatment with citalopram on plasma levels

Obviously, following chronic saline treatment, a challenge with saline did not have any effect plasma levels of citalopram. An acute challenge of citalopram increased levels to 2.26 ± 0.28 μM ($P<0.0001$). A 48 hour washout period following a 14 day treatment with citalopram was sufficient to reduce the amount of citalopram below a functional level, as plasma levels were below detection limit. Challenging the animal subsequently with citalopram raised levels to 1.91 ± 0.15 μM ($P<0.0001$). Chronic treatment without a washout resulted in plasma levels of 1.44 ± 0.05 μM, a challenge with citalopram increased plasma levels further to 3.38 ± 0.63 μM ($P = 0.0401$). Plasma levels of citalopram following either acute administration or prolonged treatment without washout are comparable. As pharmacokinetics do not differ, group effects should be attributed to treatment duration.

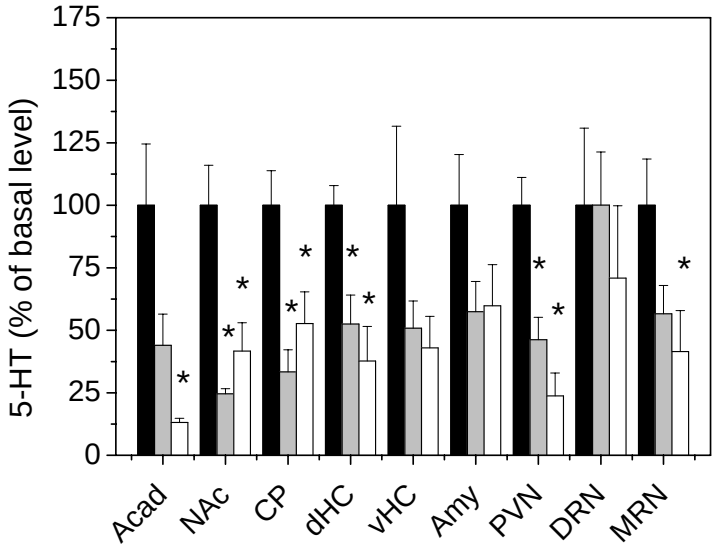

Fig. 1. *Effect of chronic treatment on intracellular serotonin stores*
*Black bars; saline treatment, saline challenge. Grey bars; citalopram treatment, 48 hour washout, saline challenge. White bars; citalopram treatment, no washout, saline challenge. * P < 0.05 versus saline treatment.*

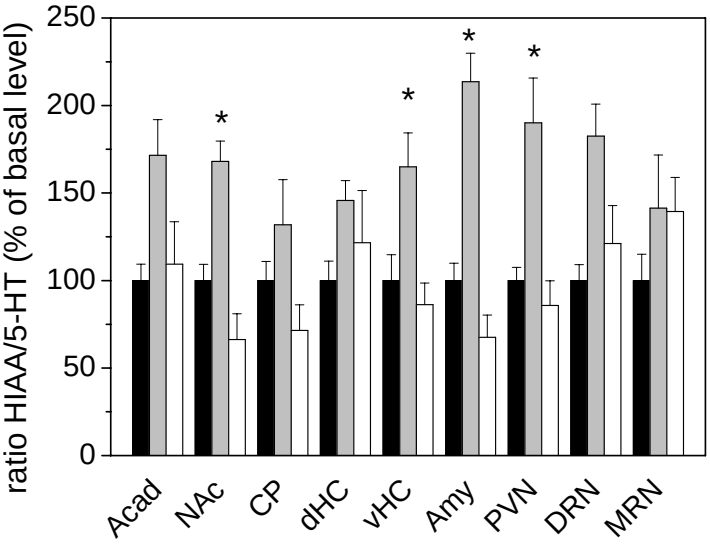

Fig. 2. *Effect of washout on serotonin turnover*
*Black bars; saline treatment, saline challenge. Grey bars; citalopram treatment, 48 hour washout, saline challenge. White bars, citalopram treatment, no washout, saline challenge. * P < 0.05 washout versus no washout.*

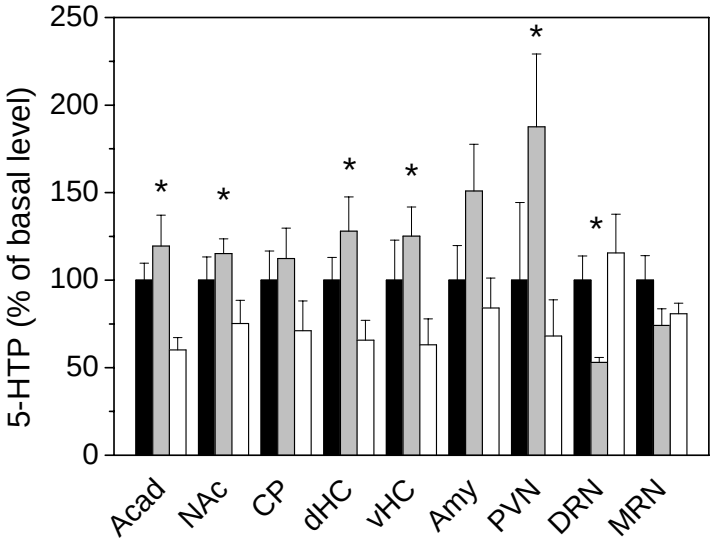

Fig. 3. *Effect of washout on serotonin synthesis*
*Black bars; saline treatment, saline challenge. Grey bars; citalopram treatment, 48 hour washout, saline challenge. White bars, citalopram treatment, no washout, saline challenge. * P < 0.05 washout versus no washout.*

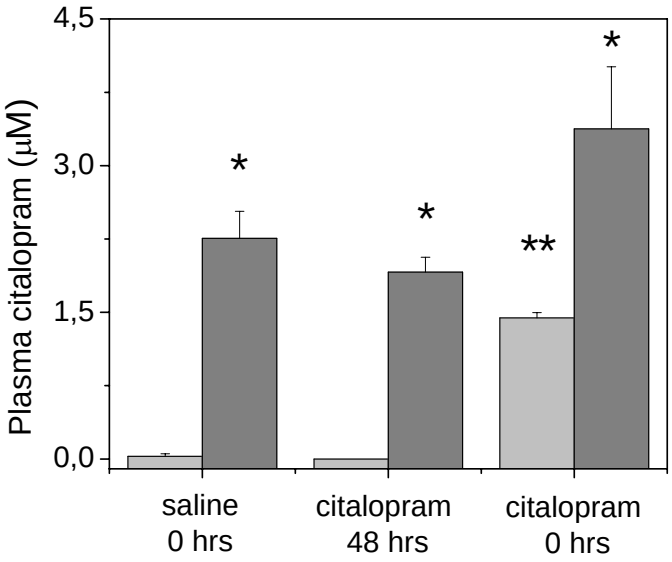

Fig. 4. *Effect acute and chronic treatment on plasma levels of citalopram*
Light grey bars; saline challenge. Dark grey bars; citalopram challenge.
** P < 0.05 versus saline challenge, ** P < 0.05 versus other saline challenged groups. Saline 0 hrs = 14 day saline treatment; citalopram 48 hrs = 14 day citalopram treatment with 48 washout; citalopram 0 hrs = 14 day citalopram treatment with no washout.*

treatment	*saline*		*saline*		*saline*		*citalopram*		*citalopram*	
washout	*0 hrs*		*0 hrs*		*0 hrs*		*48 hrs*		*0 hrs*	
challenge	*saline*		*saline*		*citalopram*		*saline*		*saline*	
5-HT	***fmol***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***
Acad	780,1 ±	191,4	100,0 ±	24,5	55,7 ±	6,5	51,2 ±	12,5	13,2 ±	1,7
Nac	663,8 ±	106,5	100,0 ±	16,0	90,1 ±	22,7	25,1 ±	2,5	41,7 ±	11,3
CP	370,6 ±	51,2	100,0 ±	13,8	51,9 ±	12,9	37,5 ±	9,5	52,7 ±	12,7
Amy	2059,8 ±	417,3	100,0 ±	20,3	67,5 ±	15,1	68,1 ±	7,3	59,8 ±	16,5
dHC	546,0 ±	43,1	100,0 ±	7,9	57,7 ±	4,2	63,4 ±	5,1	37,7 ±	13,8
PVN	1084,1 ±	121,0	100,0 ±	11,2	61,7 ±	3,9	55,1 ±	1,1	23,8 ±	9,1
vHC	1407,8 ±	445,2	100,0 ±	31,6	37,7 ±	9,7	61,5 ±	2,9	43,0 ±	12,6
DR	1676,5 ±	517,2	100,0 ±	30,8	95,9 ±	10,5	120,6 ±	6,9	70,9 ±	28,9
MRN	1328,5 ±	245,2	100,0 ±	18,5	92,2 ±	0,6	66,2 ±	7,9	41,5 ±	16,4
HIAA	***fmol***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***
Acad	483,7 ±	93,1	100,0 ±	19,3	61,4 ±	12,3	80,5 ±	17,9	18,3 ±	5,2
Nac	666,6 ±	145,7	100,0 ±	21,9	49,5 ±	8,0	48,6 ±	6,0	31,8 ±	4,1
CP	543,0 ±	98,0	100,0 ±	18,0	36,5 ±	3,8	44,7 ±	5,9	38,1 ±	1,8
Amy	557,1 ±	158,5	100,0 ±	28,5	79,8 ±	21,4	137,1 ±	32,0	42,0 ±	4,1
dHC	546,3 ±	91,9	100,0 ±	16,8	47,1 ±	5,0	87,1 ±	19,7	47,0 ±	7,2
PVN	310,6 ±	67,7	100,0 ±	21,8	71,6 ±	23,1	103,6 ±	21,4	25,8 ±	7,2
vHC	542,0 ±	141,3	100,0 ±	26,1	41,6 ±	6,2	130,6 ±	13,4	40,9 ±	5,0
DR	1019,0 ±	241,2	100,0 ±	23,7	50,7 ±	13,1	196,6 ±	13,8	58,9 ±	20,5
MRN	1072,0 ±	245,4	100,0 ±	22,9	45,9 ±	13,9	118,3 ±	31,7	55,2 ±	11,6
Turnover	***ratio***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***
Acad	0,765 ±	0,07	100,0 ±	9,34	74,7 ±	10,6	171,6 ±	20,4	109,4 ±	24,2
Nac	1,163 ±	0,11	100,0 ±	9,24	52,6 ±	6,8	168,1 ±	17,6	66,3 ±	14,7
CP	1,639 ±	0,18	100,0 ±	10,91	67,4 ±	8,2	131,9 ±	8,4	71,5 ±	14,7
Amy	0,321 ±	0,03	100,0 ±	9,91	98,5 ±	19,6	213,6 ±	16,5	67,6 ±	12,8
dHC	1,121 ±	0,12	100,0 ±	11,12	74,2 ±	8,4	145,8 ±	12,7	121,7 ±	29,7
PVN	0,330 ±	0,02	100,0 ±	7,55	70,6 ±	14,8	190,1 ±	6,8	85,7 ±	14,2
vHC	0,499 ±	0,07	100,0 ±	14,76	102,1 ±	24,0	164,9 ±	11,0	86,2 ±	12,4
DR	0,550 ±	0,05	100,0 ±	9,09	83,2 ±	9,9	182,5 ±	13,2	121,2 ±	21,7
MRN	0,949 ±	0,14	100,0 ±	15,02	63,2 ±	1,8	141,4 ±	37,7	139,4 ±	19,6
5-HTP	***fmol***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***	***%***	***SEM***
Acad	102,8 ±	9,96	100,0 ±	9,69	66,9 ±	12,9	119,4 ±	17,7	60,1 ±	7,1
Nac	97,9 ±	43,48	100,0 ±	44,41	82,7 ±	18,2	187,6 ±	41,6	68,1 ±	20,6
CP	176,4 ±	29,35	100,0 ±	16,64	110,1 ±	24,5	112,4 ±	17,3	71,1 ±	17,1
Amy	83,8 ±	10,90	100,0 ±	13,01	64,2 ±	16,0	128,0 ±	19,6	65,7 ±	11,4
dHC	195,7 ±	44,74	100,0 ±	22,86	62,6 ±	10,8	125,2 ±	16,7	63,1 ±	14,8
PVN	218,9 ±	43,22	100,0 ±	19,75	93,5 ±	6,0	150,9 ±	26,6	84,1 ±	17,0
vHC	111,7 ±	14,82	100,0 ±	13,27	85,7 ±	12,7	115,2 ±	8,4	75,2 ±	13,3
DR	2178,4 ±	301,20	100,0 ±	13,83	83,8 ±	10,2	53,1 ±	2,7	115,6 ±	22,1
MRN	1328,9 ±	185,41	100,0 ±	13,95	70,2 ±	8,1	74,2 ±	9,4	80,8 ±	6,0

Table 1. *Effect of acute and chronic treatment with citalopram in absence and presence of a washout period. Values of the control group (saline treated, saline challenge) are presented both in amount and % of basal level, values of all other groups are presented as % of control group (basal level).*

4. Discussion

The present study confirms the general observation that synthesis, release and metabolism of serotonin are all diminished in response to acute antidepressant treatment, but steadily revert to normal levels following chronic treatment. This reduced functionality of inhibitory feedback mechanisms is commonly explained by a gradual desensitization of the serotonergic autoreceptors. However, in contrast with these observations, our data indicate that intracellular serotonin remains decreased even upon chronic treatment, both after washout or in presence of citalopram. The amount of serotonin stored intracellular depends on both synthesis and reuptake of previously released serotonin. Theoretically, if synthesis cannot keep up with the conditions of chronic reuptake inhibition as induced during prolonged treatment with SSRIs, depletion of these stores could occur. Previous studies report restored (Esteban et al., 1999;Stenfors and Ross, 2002) or even increased levels of 5-HTP following chronic treatment (Moret and Briley, 1992), suggesting adaptation. However, in all cases a washout period was included which might have interfered. Like treatment itself, a certain period of drug absence after treatment could also induce pharmacological changes on the cellular level. During a washout period, resensitization or adaptation can take place, altering or even reversing the effects of chronic treatment (Neumaier et al., 1996). This is indeed confirmed by our own results, as both turnover and 5-HTP are increased after washout but showed opposite effects if no washout was included. In addition, storage was only further decreased upon treatment, which can be better explained by a simultaneous reduction in synthesis too. So arguably, the situation without washout, in presence of citalopram, more accurately depicts the neurochemical effects of the chronic treatment itself. Clinically, this is interesting too, as the pharmacological situation in presence of citalopram more closely resembles the clinical situation.

Introducing a washout out period could bear some similarity with the clinical effect known as rebound depression. When suddenly discontinuing antidepressant therapy, patients have been reported to relapse into a depressive state, suffering from an immediate reversal of all therapeutic effects. In the present study, this is resembled by the situation after a washout, which shows the effects of a sudden discontinuation of treatment rather than the effect of the treatment itself. This is most obviously seen in the amygdala, thalamus and forebrain regions. Interestingly, these areas are reported to have a high density of 5-HT_{1B} receptors, which control serotonin release and synthesis. In contrast to 5-HT_{1A} receptors, these receptors do not desensitize, so under contitions of increased extracellular serotonin, both synthesis and release are continuously inhibited as a result of 5-HT_{1B} receptor activation. A fall in extracellular levels due to sudden treatment discontinuation will reverse this process. The enhanced levels of 5-HIAA/5-HT ratio and 5-HTP

accumulation seen after washout indeed point at an increase in release and synthesis, respectively. This process might very well explain the clinical effect known as rebound. Consequently, the present study provides the neurochemical evidence to gradually phase out antidepressant treatment in order to prevent rebound effects.

But almost as important as this finding, our observations also indicate that intracellular serotonin stores in the brain are slowly depleted during chronic treatment. Although it sounds rather alarming, the clinical interpretation of these results remains unclear. If the therapeutic effect of antidepressants should be assigned to increased levels of extracellular 5-HT, it seems to be a paradox that the total amount of brain serotonin gets depleted, which is a rather unwanted side-effect in this case. On the other hand, it might be that the neurochemical basis of the therapeutic effect is not restricted to the extracellular level. By adjusting both metabolism and synthesis, the resetting of the serotonergic system as a whole could also attribute to therapeutic success.

From the present study it can be concluded that, although a washout period after chronic treatment is generally thought to be essential in order to prevent pharmacological interference, it is the washout period itself that interferes with the effect of the chronic treatment. The reversal of effects observed after a washout might refer to the rebound effect seen in the clinic and supports a gradual discontinuation to prevent a relapse.

It should also be noted that as a result of continuous reuptake inhibition and a decreased synthesis rate, intracellular serotonin stores are steadily depleted upon treatment. Further research should reveal how this affects the therapeutic effect of SSRIs as it is still unknown how to interprete this on a clinical level.

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CHAPTER 7

Summary and general discussion

Summary and general discussion

A meta-analysis of clinical studies involving the six most widely prescribed antidepressants approved between 1987 and 1999 by the FDA suggested that antidepressant treatment is only marginally more effective than placebo (Kirsch et al., 2002). Other worrying aspects of antidepressant treatments are the considerable non-response rates (30-40%) and the late onset of action (2-5 weeks). According to the World Health Organization major depression is likely to become the most frequently occurring disabling disease of the western world in the coming decade. It can therefore hardly be disputed that there is an urgent need for improved antidepressant treatment. There are, however, no signs that a major breakthrough in pharmacotherapy is to be expected in the near future. Serendipitous findings aside, such breakthrough would need a revolutionary new and scientifically verified framework of depression. Neuroimaging, genomics and proteomics are likely to form important building blocks for such a framework, but as yet our level of insight into the pathophysiology of affective disorders is insufficient to realistically expect major advances in rational drug design. Besides major depression may be too heterogeneous a disease to expect miracles from a single novel pharmacological mechanism. A more realistic approach to improve antidepressant treatment may be found in further exploring the existing hypotheses regarding major depression. Development of the majority of antidepressant drugs has been inspired by the monoamine hypothesis, which was based on serendipitous findings more than forty years ago. The hypothesis has been challenged in the past, but recent evidence from neuroimaging and DNA research support the idea that it still has considerable potential.

An approach to improve antidepressant treatment by further exploring the monoamine hypothesis is augmentation strategies. As detailed in **chapter one** several forms of augmentation are imaginable, but the present thesis mainly concerns those strategies that are aimed to further increase the effect of SSRIs on extracellular serotonin levels, by making use of antagonists of 5-HT receptors involved in inhibitory feedback mechanisms. One may question the relevance of further increasing 5-HT levels for antidepressant activity. However, striving for increased 5-HT levels is a logical consequence of Blier's desensitization hypothesis and it also fits in the monoamine hypothesis of depression. On the other hand, direct evidence for a relation between increased 5-HT levels and antidepressant activity is as yet missing. Confounding factors are the unavailability of selective and potent 5-HT receptor antagonists for use in humans and the fact that we are not yet able to estimate 5-HT levels in humans accurately. However, such problems do not arise with studies in laboratory animals.

In the present thesis an attempt has been made to address a number of important questions that can be raised with augmentation strategies. For instance, does SSRI augmentation lead to increased neuronal activity in brain areas that have been associated with major depression? In **chapter two** the expression of the immediate early gene *cfos* is used to assess the neuronal activation pattern elicited by a single dose of the SSRI citalopram both in absence and presence of the 5-HT_{1A} receptor antagonist WAY 100635. However, the results did not exactly meet the expectations. For instance, the pattern of c-Fos expression in the rat brain following the administration of citalopram did not correspond with the distribution of a particular 5-HT receptor type (Kilpatrick et al., 1987; Morilak et al., 1993; Pazos et al., 1985; Pazos and Palacios, 1985; Ward et al., 1995) or with the density of 5-HT containing nerve terminals (Steinbusch, 1981). In retrospect, however, this could be an important finding indicating that the activation of various brain areas by an SSRI does not critically depend on the activation of particular 5-HT receptor subtypes. Or to put it bluntly: What would be the use of SSRI augmentation strategies when the 5-HT receptor antagonist concomitantly inhibits the neuronal activation of brain areas involved in the antidepressant effect of SSRIs? The study also failed to demonstrate an augmented c-Fos response with WAY 100635 in several important brain areas such as prefrontal cortex, hippocampus, dorsal raphe nucleus and median raphe nucleus, which is at variance with 5-HT microdialysis studies. On the other hand, a significant augmentation was seen in the amygdala, nucleus accumbens and paraventricular nucleus of the hypothalamus, key areas of the limbic-hypothalamic-pituitary-adrenocortical system. This could indicate that, in contrast to prefrontal cortex and hippocampus, activation of postsynaptic 5-HT_{1A} receptors in these areas does not play a decisive role in the effects of SSRIs. It is also noteworthy that the mood-stabilizing effect of antidepressants has been hypothesized to result from their action on the limbic-hypothalamic-pituitary-adrenocortical system (Barden et al., 1995).

The desensitization hypothesis by Blier et al. provides a firm basis for connecting onset of action of antidepressants to a loss of 5-HT autoreceptor function. The idea that antidepressant response might be hastened by blocking the autoreceptors sounds convincing, but one may question whether it is realistic to expect enhanced efficacy when autoreceptor function is also disabled following chronic treatment with SSRIs. Many studies have reported desensitization of 5-HT_{1A} autoreceptors following chronic antidepressant treatment, indicating that there may be little to gain in terms of efficacy. In contrast to 5-HT_{1A} autoreceptors, chronic antidepressant treatment via osmotic mini-pumps did not lead to desensitization of 5-HT_{1B} autoreceptors (Cremers et al., 2000). An in situ-hybridization study has, however, shown a decreased expression of the 5-HT_{1B} receptor gene following chronic treatment, but also a rapid reversal of the effect after

discontinuation of the antidepressant (Neumaier et al., 2002). In **chapter three** it is shown that augmentation with 5-HT_{1B} receptor antagonists does not change *during* chronic antidepressant treatment, while stress markers such as corticosteron, adrenaline and noradrenaline in blood were significantly decreased (Jongsma et al., 2005). The latter effect is in accordance with both preclinical data (Jensen et al., 1999; Reul et al., 1993) and the clinical observation that chronic treatment with antidepressants restores HPA-axis hyperactivity in depressive patients (Barden et al., 1995; Inder et al., 2001) The study also indicates that 5-HT_{1B} autoreceptors do not desensitize during chronic antidepressant treatment, which makes it rather unlikely that they are actively involved in the altered stress-hormone response. This might be clinically relevant, because it suggests that the therapeutic effect of ongoing antidepressant treatment could be further improved by co-administration of a 5-HT_{1B} receptor antagonist. A confounder could be the role of postsynaptic 5-HT_{1B} receptors in the treatment of depression. It can be argued, however, that chronic antidepressant treatment desensitizes postsynaptic but not presynaptic 5-HT_{1B} receptors. Because the in situ-hybridization is not likely to discriminate between pre and postsynaptic 5-HT_{1B} receptors, and 5-HT microdialysis studies measure presynaptic effects only, this might explain the different outcome of these studies. If postsynaptic 5-HT_{1B} receptors do indeed desensitize following chronic treatment it would be rather unlikely that their activation contributes to the antidepressant effect.

Blier's desensitization hypothesis is based on data from electrophysiology studies into presynaptic 5-HT_{1A} receptors located in the dorsal raphe nucleus. However, dynamic changes of 5-HT_{1A} receptor function may not be restricted to the presynaptic receptors. Previous studies have shown that postsynaptic 5-HT_{1A} receptors located in the central nucleus of the amygdala do not only control presynaptic release but also desensitize upon chronic SSRI treatment (Bosker et al., 1997; Bosker et al., 2001). In **chapter three** it is shown that postsynaptic 5-HT_{1A} receptors in the prefrontal cortex control local release as well as 5-HT release in the serotonergic dorsal raphe nucleus, suggesting a long loop type of feedback from the prefrontal cortex to the dorsal raphe nucleus. Whereas presynaptic 5-HT_{1A} receptors in the dorsal raphe and postsynaptic 5-HT_{1A} receptors in the amygdala desensitize, the sensitivity of 5-HT_{1A} receptors in the prefrontal cortex appears to increase upon chronic SSRI treatment, which is in agreement with the observed trend toward increased [^{3}H]-8-OH-DPAT binding in the prefrontal cortex. Importantly, opposite effects on pre and postsynaptic 5-HT_{1A} receptors following chronic antidepressant treatment have also been reported by a recent study wherein an increased and decreased agonist stimulated GTPγS binding was found in hippocampus and raphe nucleus, respectively (Castro et al., 2003). Such opposite effects on pre and postsynaptic 5-HT_{1A} receptor-mediated feedback would imply a

shift in control of terminal 5-HT release from the autoreceptors to their postsynaptic counterparts, which could be a factor in the clinical efficacy of antidepressants.

The release of 5-HT and hence also the SSRI induced increases of extracellular 5-HT levels depend on 5-HT autoreceptor control, but also on the availability of the serotonin precursor tryptophan. The latter is in accordance with the clinical observation that depressed patients that were successfully treated with antidepressants suffer from a relapse following tryptophan depletion. In **chapter five,** it is shown that depletion of tryptophan or inhibition of serotonin synthesis by NSD 1015 strongly reduces the effect of an SSRI on extracellular 5-HT levels, indicating that the effect of SSRIs largely depends on precursor availability and thus *de novo* synthesis of serotonin. It is therefore conceivable that insufficient synthesis of serotonin, for instance caused by an unfavorable tryptophan hydroxylase gene polymorphism (Zhang et al., 2004; Zhang et al., 2005; Zill et al., 2004), can contribute to the high non-response rates with SSRI treatment. The importance of *de novo* serotonin synthesis is further emphasized by the additional increase of extracellular 5-HT levels observed when tryptophan is co-administered with an SSRI (van der Stelt et al., 2004, this thesis). While in most clinical studies rather high dosages of tryptophan are used, the results from the present study indicate that even modest increases of tryptophan plasma levels markedly augment the effect of an SSRI on extracellular 5-HT levels. Arguably, a lower dose of tryptophan may have a comparable effect on antidepressant activity, but it could be less prone to causing serious side effects such as the serotonergic syndrome.

It is also conceivable that SSRI augmentation with a 5-HT receptor antagonist has little effect if the lack of therapeutic effect originates from insufficient levels of tryptophan. However, while the concept of antagonist-based augmentation has been applied clinically by co-administration of an SSRI with the mixed β-adrenergic and 5-HT $_{1A}$ receptor antagonist pindolol (Ballesteros and Callado, 2004) or the 5-HT$_{2C}$ receptor antagonist mianserin (Maes et al., 1999; Ferreri et al., 2001), no attention has been paid in those studies to the circulating tryptophan levels. In **chapter five** the effect of tryptophan supplementation on 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{2C}$ receptor antagonist based augmentation strategies is also investigated. While tryptophan further increased all three forms of 5-HT antagonist-based SSRI augmentation, only antagonism of 5-HT$_{1B}$ receptors was capable to significantly enhance the augmentation observed with tryptophan. Apparently, when the precursor is sufficiently present, 5-HT$_{1B}$ receptor-controlled processes like synthesis and release become the limiting factor. Summarizing, it should be taken into account that if treatment with serotonergic antidepressants is insufficient even following augmentation, additional tryptophan might be required to attain the therapeutic effect.

Release of 5-HT and the effect of SSRIs on extracellular 5-HT levels are limited by the availability of tryptophan, but otherwise depend on intracellular serotonin stores. These stores rely both on synthesis and reuptake of previously released serotonin. So theoretically, under conditions of prolonged reuptake inhibition, synthesis needs to adjust in order to prevent depletion of intracellular serotonin stores. In **chapter six** it is shown that as a result of continuous reuptake inhibition and decreased synthesis (through the activation of the autoreceptors) intracellular serotonin stores are steadily depleted. Future research should reveal whether this could be part of the mechanism of action or must be regarded as an unwanted side effect of SSRIs.

Another worrying aspect of chronic antidepressant treatment is the clinical phenomenon called rebound depression. When antidepressant therapy is suddenly discontinued, patients have been reported to relapse into a depressive state, emphasizing the need to slowly phase out SSRI treatment. An analogy may be found with the washout period in preclinical chronic treatment studies, which is commonly used to avoid interference with the pharmacological probes. Arguably, the effects of a sudden discontinuation of treatment are more prominent than the effect of the treatment itself. The latter possibility is also investigated in **chapter six** by comparing the effects of chronic SSRI treatment on total serotonin content, synthesis and metabolism in presence and absence of a washout period.

The data suggest that the washout period has caused a strong increase of 5-HT metabolism, which could not be compensated by *de novo* synthesis resulting in a rather dramatic depletion of serotonin stores. Interestingly, the most marked effects were measured in brain areas with a high density of 5-HT_{1B} receptors. Arguably, the sudden discontinuation of the SSRI leads to a strong decline in 5-HT_{1B} autoreceptor activation thereby dramatically increasing 5-HT release and metabolism. This would also imply that 5-HT_{1B} receptors do not desensitize following chronic treatment, which is in line with the study in **chapter three**. The effects of the washout period on all tested parameters are indeed considerable and it is tempting to causally relate a sudden decline in 5-HT_{1B} autoreceptor activation to the phenomenon of rebound depression. Finally, the washout study clearly supports the clinical practice to gradually phase out antidepressant treatment in order to prevent rebound effects.

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SAMENVATTING

Depressie

De term depressie wordt van oudsher gebruikt voor neerslachtige gevoelens en sombere gedachten. In de oudheid werden verschillende termen gebruikt om dit syndroom te benoemen, en sedert de middeleeuwen werd vaak de term *melancholie* gebruikt om een groot scala aan symptomen samen te vatten. Er bestond toen echter geen manier om via algemeen erkende classificatiesystemen een depressie af te grenzen van andere psychiatrische syndromen, zoals angststoornissen en psychosen. Pas in de jaren 70 van de twintigste eeuw wordt het ziektebeeld depressie in de psychiatrie nauwkeuriger omschreven in classificatiesystemen zoals de Diagnostic and Statistical Manual of Mental Diseases (DSM). De diagnose *depressief syndroom* omvat een groot scala aan negatieve affectief beleefde symptomen zoals ernstige somberheid, anhedonie en schuldgevoelens. Tevens is sprake van lichamelijke symptomen zoals slaapstoornissen, afgenomen libido en verminderde eetlust. Daarnaast bestaan er meer cognitieve symptomen zoals een verminderd gevoel van eigenwaarde en suïcidale gedachten.

Alhoewel antidepressiva niet meer weg te denken zijn uit de huidige maatschappij vindt tot op heden nog steeds ongeveer 30% van mensen die ze krijgen voorgeschreven hier geen baat bij. Een geneesmiddel dat slechts in 70% van de gevallen effectief is, is op zijn minst zeer matig werkzaam te noemen en vraagt om verbetering.

De geschiedenis van antidepressiva

Antidepressiva zijn per toeval ontdekt en deze toevalligheid is nog steeds de basis voor het ontwikkelen van nieuwe geneesmiddelen tegen depressie.

Ergens in de jaren 50 bleek dat iproniazide, een destijds veel gebruikt geneesmiddel tegen tuberculose, ook een positief effect had op de gemoedstoestand van met iproniazide behandelde tuberculose patiënten. Het bleek de hoeveelheid serotonine in de hersenen te verhogen. Een decennium daarvoor werd serotonine in het bloed ontdekt, een stof die, zoals de naam al doet vermoeden, de bloedvaten bleek samen te trekken. De drug LSD die dit effect kon tegengaan, had tevens een sterk effect op de gemoedstoestand. Dit leidde tot het vermoeden dat de stof serotonine ook aanwezig is in de hersenen en betrokken is bij het reguleren van de stemming.

Deze feiten hebben geleid tot de hypothese dat depressie samenhangt met een tekort aan serotonine en dat het verhogen van serotonine in de hersenen leidt tot een verbetering van de

stemming. Tot op de dag van vandaag vormt deze hypothese het uitgangspunt voor het verder ontwikkelen en het verbeteren van geneesmiddelen tegen depressie. Het lijkt misschien wat achterhaald om onderzoek te verrichten op basis van een hypothese van ruim 40 jaar oud. Uit recenter onderzoek blijkt echter dat er wel degelijk een relatie is tussen serotonine en depressie.
In de jaren 90 is onderzoek gedaan naar tryptofaan, de bouwsteen van serotonine. Voor het aanmaken van serotonine in de hersenen is deze stof essentieel. Depressieve patiënten die succesvol behandeld waren met antidepressiva, werden weer depressief wanneer ze niet genoeg tryptofaan kregen. Voor de werking van antidepressiva is het aanmaken van voldoende serotonine en dus de aanwezigheid van genoeg tryptofaan van essentiëel belang. In een andere studie werd aangetoond dat mensen die door een genetisch defect minder goed serotonine kunnen aanmaken een hogere kans hebben op depressie. Deze recentere onderzoeksresultaten ondersteunen de eerder genoemde serotonine hypothese.
Ongeacht de wetenschappelijke evidentie voor het bestaan van een relatie tussen serotonine en depressie, is het nog steeds niet duidelijk hoe een verhoging van de serotoninespiegel in de hersenen, zoals veroorzaakt door antidepressiva, samenhangt met een antidepressief effect.

Hoe werkt serotonine?

De chemische benaming voor de stof serotonine is 5-hydroxy tryptamine, 5-HT. Deze afkorting wordt veel gebruikt in de literatuur en ook in dit proefschrift. Serotonine is een signaalstof, een neurotransmitter. Neurotransmitters worden uitgescheiden door de uitlopers van zenuwcellen en weer opgenomen door de zenuwcel die tegen de uitloper aanligt. De zenuwcellen vormen met hun uitlopers een heel netwerk. Door neurotransmitters uit te scheiden in de synaps, de ruimte tussen de ontvangende cel en de uitloper van de signaalcel, geven deze cellen signalen aan elkaar door. Receptoren op de ontvangende zenuwcel kunnen het signaal beïnvloeden. Elk neurotransmittersysteem kent een aantal receptoren die, afhankelijk van hun werking, het doorgeven van het signaal kunnen manipuleren. Voor serotonine zijn een 14-tal 5-HT receptoren bekend die elk op hun eigen manier het serotonerg systeem kunnen beïnvloeden. Ook op het effect van antidepressiva zijn deze 5-HT receptoren van invloed. In dit proefschrift komen daar drie van aan bod: de 5-$HT_{1A, 1B}$ en $_{2C}$ receptoren.
De serotonerge zenuwcellen liggen geklusterd in een kerngebiedje in de hersenstam, de raphe-kernen, en hebben hun uitlopers, de axonen, naar allerlei hoger gelegen hersengebieden. Deze serotonerg aangestuurde gebieden vormen tezamen het limbisch systeem dat als taak heeft emoties en gevoelens te reguleren. Het verminderd functioneren van het limbisch systeem wordt verondersteld een rol te spelen bij depressie en het ontstaan daarvan.

Serotonine en antidepressiva

Alhoewel dus bij toeval ontdekt, bleek het eerste antidepressivum iproniazide inderdaad de hoeveelheid serotonine in de hersenen te verhogen. Dit effect berust op remming van een enzym, monoamine oxidase, dat serotonine in de synaps afbreekt en kenmerkt de eerste generatie antidepressiva, de monoamine oxidase remmers, de MAO's. Al vrij snel hierna werd een volgende generatie antidepressiva ontwikkeld, de tricyclische antidepressiva, of TCA's. De werking van TCA's berust op het feit dat zenuwcellen de uitgestoten neurotransmitters na gebruik opnieuw op kunnen nemen. Door deze heropname te remmen kunnen de tricyclische antidepressiva de hoeveelheid neurotransmitter in de synaps tijdelijk verhogen. Helaas bleken beide generaties antidepressiva niet erg selectief, want niet alleen serotonine werd verhoogd maar ook aanverwante neurotransmitters. Dit resulteerde in lastige en in veel gevallen ook ernstige bijwerkingen. Omdat TCA's wel effectieve antidepressiva zijn, is bij de nieuwe, derde generatie antidepressiva opnieuw gebruik gemaakt van heropname remming, maar om bijwerkingen tegen te gaan dit keer selectief voor serotonine. De ontwikkeling van de selectieve serotonine heropname remmers, de SSRI's, was hiermee een feit geworden. Tot deze generatie horen onder andere de populaire antidepressiva Prozac (fluoxetine), Effexor (venlafaxine), Seroxat (paroxetine) en Citalopram (Cipramil). Ondanks het feit dat de werking van de SSRI's niet verbeterd is ten opzichte van hun voorgangers, heeft de vermindering van de bijwerkingen er voor gezorgd dat ze nu tot de meest voorgeschreven klasse antidepressiva behoren.

Antidepressief effect

Merkwaardig genoeg duurt het een week of vier voordat antidepressiva goed werken. Dit is opmerkelijk omdat uit onderzoek blijkt dat de heropname van 5-HT al meteen na de eerste toediening van de SSRI geremd wordt. De verhoging van serotonine die hierdoor ontstaat wordt echter meteen weer tegengegaan doordat de extra serotonine zich bindt aan de 5-HT receptoren. Dit heeft juist een remmend effect op het serotonerg systeem. Uit studies met proefdieren blijkt dat na langdurige behandeling sommige 5-HT receptoren minder gevoelig worden waardoor het remmend effect op serotonine ook vermindert. Opvallend genoeg komt deze tijdsduur goed overeen met de periode van ongeveer vier weken voor het effectief worden van antidepressiva. Hieruit is dan ook de conclusie getrokken dat het antidepressief effect pas optreedt nadat gevoeligheid van de 5-HT receptoren is verminderd omdat dan pas serotonine niet langer geremd wordt. Voor deze theorie van de verminderde gevoeligheid wordt ook wel de term desensitisatie gebruikt.

Nieuwe ontwikkelingen

Een snellere werking van antidepressiva zou bewerkstelligd kunnen worden door de 5-HT receptoren meteen te blokkeren in plaats van te wachten op verminderde gevoeligheid. Van deze gedachte is gebruik gemaakt in de augmentatiestrategie; de werking van een SSRI wordt verbeterd door tegelijktijdig een blokker toe te dienen: een 5-HT receptor antagonist. De extra verhoging van 5-HT (augmentatie) gemeten in proefdieren die een 5-HT receptorblokker kregen toegevoegd tijdens behandeling met een SSRI doet vermoeden dat dit idee heel goed blijkt te werken. Helaas kwam uit klinische studies niet een eenduidige verbetering van het antidepressieve effect naar voren. Toch wordt augmentatie wel als veelbelovend concept beschouwd in de speurtocht naar verbeterde werking van de huidige antidepressiva. Zoals de titel van dit proefschrift al doet vermoeden worden in dit proefschrift de mogelijkheden en de beperkingen besproken van serotonerge augmentatie strategieën.

Onderzoek in dit proefschrift

Voor het meten van de hoeveelheid serotonine in de synaps is in het onderzoek beschreven in dit proefschrift gebruik gemaakt van de microdialyse techniek. In de hersenen van de rat wordt een klein buisje met een membraantje ingebracht van minder dan een halve millimeter dik. Serotonine trekt vanuit de hersenen door het membraantje heen en wordt er vervolgens uit gespoeld. Zo kunnen veranderingen in de hoeveelheid 5-HT rechtstreeks worden gemeten. Het voordeel van deze techniek is dat er in de hersenen van levende proefdieren kan worden gekeken naar de effecten van geneesmiddelen. De dieren zelf merken overigens weinig van deze manier van meten en worden geopereerd onder volledige narcose. Het nadeel van deze techniek is dat je op maar één of twee plaatsen tegelijk in de hersenen kan meten. Het moet dus wel van tevoren duidelijk zijn waar in de hersenen een effect te verwachten is. In **hoofdstuk twee** is daarom bestudeerd welke gebieden in de hersenen actief worden na toediening van een SSRI, en ook of er een verandering in dat patroon ontstaat door augmentatie met een blokker van de 5-HT_{1A} receptor.

Voor het opsporen van geactiveerde zenuwcellen is het eiwit c-Fos gebruikt. Dit eiwit is gemakkelijk te kleuren en verschaft informatie over de activiteit van de betreffende cel.

Het merkwaardige was dat de hersengebieden waar met microdialyse veel verhoging van serotonine werd gemeten, weinig geactiveerde zenuwcellen bevatten. Het is dus de vraag of een verhoging van serotonine wel een effect heeft op de activiteit van de naburige cel. Een tweede vraag is welke manier van meten het beste is om het antidepressief effect te meten: microdialyse of c-fos. Moeten we kijken naar neurotransmitter afgifte of juist naar activatie van zenuwcellen?

In **hoofdstuk drie** wordt beschreven wat het effect is van een langdurige behandeling met een SSRI op de gevoeligheid van de 5-HT_{1A} receptor. Deze receptor zit uitsluitend op het cellichaam van de zenuwcel, in tegenstelling tot de 5-HT_{1B} receptor die juist alleen maar op de zenuwuiteinden gevestigd is. Voor de 5-HT_{1A} receptoren is aangetoond dat deze desensitiseren na langdurige SSRI behandeling. Deze metingen hebben echter alleen plaatsgevonden in de serotonerge raphe-kernen, omdat hier de meeste cellichamen en dus ook veel 5-HT_{1A} receptoren te vinden zijn. Maar ook elders in de hersenen zijn veel van deze receptoren te vinden en het is de vraag of die ook verminderd gevoelig kunnen worden. In hoofdstuk drie is gemeten in de prefrontale cortex. Omdat depressieve patiënten een sterk verminderde activiteit in de cortex hebben is dit een interessant gebied voor onderzoek naar de effecten van antidepressiva. In tegenstelling tot wat er eerder in de raphe-kernen is gevonden, blijken de 5-HT_{1A} receptoren in dit gebied juist gevoeliger te worden door langdurige behandeling. Dit suggereert dat er heel anders naar het tot stand komen van het antidepressief effect moet worden gekeken; want misschien speelt juist de verhoogde gevoeligheid in de cortex een belangrijke rol hierbij. Dit betekent dus ook dat er in de hersenen geen eenduidige biologische respons hoeft te bestaan tijdens behandeling met antidepressiva, maar dat er regionale verschillen bestaan die gezamenlijk het effect kunnen verklaren.

Ook naar de gevoeligheid van de 5-HT_{1B} receptor na langdurige SSRI behandeling is veel onderzoek gedaan. In tegenstelling tot de 5-HT_{1A} receptor zijn de resultaten hiervan niet erg eenduidig; sommige onderzoekers vinden een verminderde gevoeligheid, andere zien geen effect. Dit kan te maken hebben met de duur van de behandeling; in veel gevallen wordt de behandeling een paar dagen voor het onderzoek gestopt om te zorgen dat er geen actieve stof meer in het proefdier zit als er wordt gemeten. Dit is weliswaar een logische gedachte, maar het kan het de onderzoeker ook op het verkeerde been zetten. Uit onderzoek is namelijk gebleken dat veranderingen in de gevoeligheid van 5-HT_{1B} receptoren snel kunnen herstellen na het stoppen van de behandeling. Wanneer men het antidepressivum te lang laat uitwassen kan in die tijd herstel optreden van de gevoeligheid en is verandering niet meer te meten. Om dit te voorkomen is in **hoofdstuk vier** gemeten terwijl het antidepressivum nog aanwezig was. Dit is bovendien ook beter te vergelijken met de werkelijke klinische situatie. We willen immers weten wat er gebeurt wanneer je het antidepressivum geeft en niet wat er gebeurt als het een poosje wel en dan weer niet gegeven wordt. Ook in aanwezigheid van de SSRI verandert de gevoeligheid van de 5-HT_{1B} receptor niet. Dus in tegenstelling tot de 5-HT_{1A} receptor blijft deze gedurende de behandeling de serotonerge activiteit onderdrukken. Misschien is het dus wel interessant om patiënten die niet reageren op een SSRI ook te behandelen met een 5-HT_{1B} blokker teneinde zo het antidepressief effect te versterken.

Alhoewel chronische behandeling geen effect bleek te hebben op de gevoeligheid van de 5-HT_{1B} receptor, bleek de stressreactie wel te zijn verminderd. Als proefdieren langdurig blootgesteld worden aan stress ontwikkelen ze symptomen die sterke gelijkenis vertonen met een depressie. Ook het feit dat sommige depressieve patiënten een verhoogde hoeveelheid stress hormoon in het lichaam hebben doet vermoeden dat dit kan bijdragen aan het ontstaan van depressie. Volgens de resultaten van hoofdstuk vier blijkt langdurige behandeling met een SSRI de hoeveelheid stress hormoon in proefdieren te verlagen. Dit zou misschien ook kunnen bijdragen aan het antidepressief effect.

De afgifte van serotonine in de synaps wordt niet alleen gecontroleerd door de 5-HT receptoren, maar hangt ook af van hoeveel serotonine er wordt aangemaakt door de zenuwcel zelf. Om serotonine te maken moet de bouwsteen tryptopfaan, een essentieel aminozuur, uit de voeding worden opgenomen. Zonder tryptofaan kan er geen serotonine gemaakt worden. Hoe belangrijk tryptofaan is voor het antidepressief effect blijkt uit het feit dat succesvol behandelde patiënten weer depressief werden als ze te weinig tryptofaan kregen. Het zou natuurlijk best kunnen dat een groot aantal van de mensen die niet reageren op een antidepressivum ook te weinig tryptofaan hebben om genoeg serotonine aan te maken.

In **hoofdstuk vijf** is onderzocht wat de invloed is van tryptofaan op de werking van antidepressiva. Tryptofaan kan verlaagd worden door proefdieren een dieet zonder tryptofaan te geven. Een verlaagde hoeveelheid tryptofaan vermindert inderdaad het effect van SSRI's. Ook door de aanmaak van serotonine te remmen kan de effectiviteit van SSRI's dus worden verminderd. Hiermee is het bewijs geleverd dat, zoals eerder waargenomen bij mensen, het antidepressief effect afhangt van de beschikbare hoeveelheid tryptofaan en serotonine.

Wat gebeurt er dan als de hoeveelheid tryptofaan wordt verhoogd? Zoals te verwachten is wordt het effect van de SSRI versterkt wanneer er extra tryptofaan wordt bijgegeven. Dus niet alleen met serotonine receptor antagonisten, maar ook met extra tryptofaan kan de werking van SSRI's worden geaugmenteerd. Hoe meer tryptofaan, hoe meer augmentatie, maar de vraag is dan natuurlijk: hoeveel stijging van serotonine is nodig voor een antidepressief effect? Dat is een moeilijke vraag, die helaas nog niet te beantwoorden is. Pas als het mogelijk is om in de menselijke hersenen de hoeveelheid serotonine in de synaps te meten kunnen we daar een antwoord op gaan zoeken. Tot die tijd moeten we het doen met metingen in proefdieren.

Het effect van antidepressiva wordt meestal gemeten als hoeveelheid afgegeven serotonine in de synaps. Die serotonine komt uit blaasjes die opgeslagen zijn in de zenuwcel zelf. Deze hoeveelheid is ongeveer 1000 keer hoger dan wat er afgegeven wordt in de synaps, een soort serotonine voorraad dus. Die voorraad is afhankelijk van zowel de aanmaak van serotonine als de heropname uit de synaps. Langdurige behandeling met SSRI's blokkeert de heropname continu.

Als de aanmaak dan niet toeneemt, zal de voorraad dus verminderen. Uit de resultaten van **hoofdstuk 6** blijkt inderdaad dat door langdurige behandeling de aanmaak van serotonine duidelijk vermindert en daardoor ook de hoeveelheid serotonine in de cel sterk afneemt. Of dit moet worden gezien als een ongewenste bijwerking of juist als onderdeel van het werkingsmechanisme van SSRI's is nog niet duidelijk.

In hoofdstuk 6 komt eveneens duidelijk naar voren dat het laten uitwassen van de SSRI gevolgen heeft voor de metingen. De effecten gemeten na het uitwassen zijn tegenovergesteld aan de effecten gemeten zonder uitwassen, dus in aanwezigheid van de SSRI. Dit laatste laat zien wat het effect is van een langdurige behandeling met SSRI's, terwijl het effect gemeten na een uitwas periode een idee geeft wat de gevolgen zijn van het plotseling stoppen met een SSRI behandeling. Waarschijnlijk is dit te vergelijken met het 'rebound effect': wanneer patiënten behandeld met SSRI's plotseling stoppen met hun behandeling kunnen alle klachten acuut en in ernstige mate terugkomen. De resultaten beschreven in hoofdstuk 6 ondersteunen deze klinische bevindingen en pleiten voor een geleidelijke beïndiging van de behandeling om een rebound te voorkomen.

Conclusie

Voor het verbeteren van de huidige generatie antidepressiva worden in dit proefschrift een aantal wegen beschreven. De antidepressieve effecten van de momenteel meest voorgeschreven klasse van antidepressiva, de SSRI's, kan worden versterkt door gelijktijdige toediening met 5-HT receptor blokkers. Met name de 5-HT_{1B} receptor lijkt hiervoor een goede kandidaat omdat deze receptor niet desensitiseert. Ook door extra toediening van de voorloper van 5-HT, tryptofaan kan de werking van SSRI's worden versterkt. Met slechts een toename van 10% van tryptofaan in het bloed kan het effect van SSRI's worden verdubbeld. Voor het vertalen van deze bevindingen naar de mens is het toepassen van scan technieken bij de mens onontbeerlijk. Zodra huidige technieken het mogelijk maken serotonine in het menselijk brein te meten zal toekomstig onderzoek naar verbetering van huidige antidepressiva zich dan ook hierop moeten richten.

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